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REMARKS

Claims 27-32 are pending in this application.

A. Compliance with 35 USC 102

The Patent Office rejected Claim 27 under 35 USC 102(a) as being anticipated by Storm et al., Nature 368:639 (14 April 1994), which describes a sequence encoding a protein comprising SEQ ID NO:24 but does not describe chondrogenic activity. According to MPEP 715, a Declaration under 37 CFR 1.131 can be used to overcome a 35 USC 102(a) rejection. Attached is a copy of Declaration of Prior Invention in the United States To Overcome Cited Publication Under 37 CRF § 1.131, to swear behind Storm et al. at a date prior to 14 April 1994.

B. Compliance with 35 USC 103

The Patent Office rejected the claims under 35 USC 103(a) as being unpatentable over Storm et al., Nature 368:639 (14 April 1994), and Neidhardt PCTEP93/00350, published 19 August 1993. Under the statute, a patent may not be obtained if the difference between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made. Neidhardt is admitted not to describe a sequence encoding a protein comprising SEQ ID NO:24, only expression vectors, host cells, and processes for the production of proteins. The Rule 131 Declaration establishes prior invention prior to 14 April 1994. At the time the invention was made, Neidhardt's description of only expression vectors, host cells, and processes for the production of proteins could not have rendered the subject matter as a whole obvious because a sequence encoding a protein comprising SEQ ID NO:24 was unknown. The secondary reference does not fill in the gap left by the antedating of the primary reference, thus the rejection cannot prevail.

CONCLUSION

In view of the above, it is submitted that the claims are in condition for allowance. Reconsideration and withdrawal of all outstanding rejections are respectfully requested. Allowance of the claims at an early date is solicited. If any points remain that can be resolved by telephone, the Examiner is invited to contact the undersigned at the below-given telephone number.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: 7/8/04

By: _____

Nancy W. Vensko
Registration No. 36,298
Attorney of Record
Customer No. 45,311
(805) 547-5585

AMEND
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NIH099.001C1 PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Luyten et al.
Appl. No.	:	09/574,819
Filed	:	May 19, 2000
For	:	CARTILAGE-DERIVED MORPHOGENETIC PROTEINS
Examiner	:	Romeo, David. S.
Group Art Unit	:	1647

DECLARATION OF PRIOR INVENTION IN THE UNITED STATES TO OVERCOME CITED PUBLICATION UNDER 37 CRF § 1.131

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. This declaration is to establish completion of the invention of this application in the United States at a date prior to 14 April 1994, the date that appears on Storm et al., Nature 368:639-643 (1994), attached, which we understand was cited by the examiner. Storm et al. (Fig. 1) shows the sequence alignment of the carboxyl terminal mature domains of GDF-5 (CDMP-1), GDF-6 (CDMP-2), and GDF-7. Fig. 1 also shows the amino acid sequence of the full-length mouse GDF-5 (CDMP-1). But this is irrelevant. The claimed invention does not encompass GDF-5 (CDMP-1). Rather it encompasses GDF-6 (CDMP-2) because here is the patent claim:

An isolated DNA molecule which codes for a protein of the TGF-β family, wherein said protein has chondrogenic activity *in vivo* and comprises a sequence WIIAPLEYEAYHCEGVCDFPLRSHLEPTNHA.

2. The persons making this declaration are the named co-inventors.

3. At the time of the 7 Nov 1994 filing date of this application, bone morphogenetic proteins (BMPs) were known to be members of the TGF-β superfamily that can induce endochondral bone formation in adult animals. This superfamily includes a large group of structurally related signaling proteins that are secreted as dimers and then cleaved to result in biologically active carboxyl terminal mature domains of the protein. These bioactive proteins are characterized by 7 highly conserved cysteine residues. See, for example, Rosen V. & Theis R.S., 1992 Trends Genet. 8: 97-102 at Fig. 3, attached.

4. To establish the date of completion of the invention of this application, the following true copies are submitted as evidence: Exhibit A: Employee Invention Report signed by the co-inventors and witnessed by non-inventor witnesses, and Exhibit B: Formatted Alignment of the carboxyl terminal mature domains of CDMP-1 (designated cdmp4matORF) and CDMP-2 (designated bmp3xmatORF), both exhibits dated prior to 14 April 1994, the dates of which have been removed.

5. From Exhibit A it can be seen that partially purified extracts from newborn calf articular cartilage were found to induce cartilage and bone when subcutaneously implanted in rats. This activity showed characteristics of bone morphogenetic proteins (BMPs). Degenerate oligonucleotide primer sets derived from the highly conserved carboxyl-terminal region of the BMP family were designed and used in reverse transcription-polymerase chain reactions with poly(A)+ RNA from articular cartilage as template to determine which BMPs are produced by

chondrocytes. Two novel members of the BMP family were identified and designated cartilage-derived morphogenetic protein-1 (CDMP-1) and -2 (CDMP-2). Their carboxyl terminal mature domains revealed a common SHLEP motif, thus defining a novel subfamily. In contrast to other members of the BMP family, expression of both genes was observed predominantly in cartilaginous tissues both pre- and postnatally. Recombinant protein from one of the novel genes was expressed in COS 1 cells and demonstrated de novo cartilage inducing activity in vivo.

6. Exhibit B shows sequence alignment of the carboxyl terminal mature domains of CDMP-1 and CDMP-2, characterized by 7 highly conserved cysteine residues.

7. These results were subsequently published as Chang et al., J. Biol. Chem. 269:28227-28234 (Nov 1994), attached. By 14 April 1994, we had completed the work described in Fig. 1 (chondrogenic activity), Fig. 4 (sequence alignment), Fig. 5 (southern blot), Fig. 6 (genetic mapping), Fig. 7 (multiple tissue northern analysis), Fig. 8 (in situ hybridization), and Fig. 9 (in situ hybridization).

8. The evidence establishes completion of the claimed invention, an isolated DNA molecule which codes for a protein of the TGF-β family, wherein said protein has chondrogenic activity *in vivo* and comprises a sequence WIIAPLEYEAYHCEGVCDFPLRSHLEPTNHA, the completion being commensurate with the extent of the invention as shown in the reference Storm et al., that is, the amino acid sequence for the carboxyl terminal mature domain of GDF-6 (CDMP-2).

Appl. No. : **09/574,819**
Filed : **May 19, 2000**

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application or patent issuing therefrom.

Dated: July 5, 2004

By: _____
Frank P. Luyten, M.D.

Appl. No. : **09/574,819**
Filed : **May 19, 2000**

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application or patent issuing therefrom.

Dated: 7/3/04 By: Malcolm Moos, Jr., M.D., Ph.D.
Malcolm Moos, Jr., M.D., Ph.D.

Appl. No. : **09/574,819**
Filed : **May 19, 2000**

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application or patent issuing therefrom.

Dated: 7/6/'04 By: _____
Steven C. Chang, M.D.

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copy 1

PHS Employee Invention Report

For Patent Branch Use
E-Number
U.S.P.A.#
U.S. Filing (date)

Part I: To Be Completed by the Inventor

First Inventor: FRANK P. LUYTEN Phone 402-3502 office 496-5681 lab

1. Give a short descriptive title of your discovery or invention.

Subfamily of cartilage-derived morphogenetic proteins belonging to the TGF-beta superfamily

2. Please provide (in non-scientific terms if possible) a one paragraph description of the essence of your discovery or invention.

This subfamily of cartilage-derived morphogenetic proteins could include key molecules governing the induction and maintenance of cartilage, bone tissues, as well as the differentiation of cartilage, bone, and bone marrow.

A longer description of the invention is included as an addendum.

3. Who contributed to the invention or discovery? Please identify all colleagues who could merit co-authorship credit for the associated publication, whether or not you believe them to be "co-inventors."

Malcolm Moos, Jr, MD, PhD
A. H. Reddi, PhD
Steven Chang, medical student
Bang Hoang, medical student
Slobodan Vukicevic, MD, PhD
Ping Chen, PhD
Nick Ryba, PhD
Marie Krinks
Logan Kleinwaks

4. Is anyone outside of the Public Health Service aware of your invention or discovery? If so, please identify them and describe the dates and circumstances.

Yes, Dr. A.H. Reddi, as former mentor (since 1989): insight in the characterization of cartilage-derived, cartilage- and bone-inductive activity.

5. Are you aware of any PHS patent applications that are related to your invention or discovery?

No

6. Please list the most pertinent previous articles, presentations or other public disclosures, made by you or by other researchers, that are related to your invention or discovery. Also, attach copies, please!

No public disclosures.
For related articles, please see the addendum.

7. Please indicate any future dates on which you will publish articles or make any presentations related to your invention or discovery.

June 10, 1994

8. a. Is the subject matter of your invention related to a PHS CRADA involving your laboratory or ICD?

√ No
_ Yes. If yes, please identify the collaborator:

b. Is the subject matter based on research materials that you obtained from some other laboratory?

√ No
_ Yes. If yes, please attach any material transfer agreements (MTA) under which you received the material.

9. What companies or academic research groups are conducting similar research (if you know)? Can you identify any companies that may be good licensing prospects?

Procter & Gamble, Merck, Pfizer, Eli Lilly, Genentech, Genetics Institute, Creative Biomolecules, Advanced Tissue Engineering, and other companies invoved in treatment of skeletal diseases.

10. What further research would be necessary for commercialization of your inventions? Generally, what are your future research plans for the invention and/or for research in areas related to the invention?

Expression of recombinant protein and biological evaluation of:

1. **in vivo cartilage, bone and bone marrow induction, repair and regeneration;**
2. **developmental regulation: pattern formation and limb development.**
3. **hematopoietic, neurotrophic and myotrophic activities**

11. **First Inventor Information:** *(Provide this information for each inventor who contributed to the essence of the invention. If more than one, use Page 4, "Information on Additional Inventors.")*

Name	Degree	Social Security No.
Frank P. Luyten	MD, PhD	218-11-7975

Position Title	Office address
Visiting Scientist	Bg. 10, Rm. 1N108

Office Phone No.	FAX No.	Citizenship
402-3502	480-2012	☐ U.S. ☒ Other: Belgium

Home Address
10 Pitt Court, Rockville, MD 20850

Affiliation ☒ ICD *(specify ICD and applicable box below):* NIDR

☐ GS ☐ CO ☒ Visiting Scientist ☐ Special Volunteer
☐ GM ☐ Visiting Fellow ☐ Howard Hughes Fellow ☐ Other (specify):
☐ SES ☐ Visiting Associate ☐ Guest Researcher

☐ Non-ICD Affiliation *(specify):*

If more than one inventor, what specific contribution did you make to this work?

Overall concept, purification of bioactive proteins, cloning, expression, localization in human development

Part II: To be completed by the Technology Development Coordinator

12. Institute(s) or Agency(s) sponsoring this invention

NIDR and CBER/FDA

13. Patent prosecution fees are to be charged to

CAN:	832-4625	Costs will be shared with CBER/FDA; oral agreement with Bea	
ICD:	NIDR	Droke ; mechanism to be worked out.	

14. Inventors' Signatures

▶ This report is submitted pursuant to Executive Order 10096 and 10930 and/or Department Regulations, in order that a determination may be made as to the Government's rights and interests in the invention.

Inventors's Signatures	Dates	Witnesses's Signatures	Dates
Michael Moos Jr., MD, PhD		Jacob A. Donkersloot	
Frank P. Luyten, MD, PhD		Jacob A. Donkersloot	

15. The following Authorizing Official has *(please check the appropriate box below):*

- ☐ approved this invention for patent filing.
- ☐ recommends this invention be assigned to the inventors.
- ☐ recommends this invention be placed in the public domain.

× patentability search

Authorizing Official *(Typed)*	Signature	Date
Dr. Pamela G. Robey	Pamela G. Robey	/ /

Philip D. Noguchi, M.D.
Director, Division of Cellular and Gene Therapies
CBER, FDA

Information on Additional Inventors *(copy as needed)*

Name	Degree	Social Security No.
Malcolm Moos, Jr.	MD, PhD	471-6224-76

Position Title	Office Address
Senior Staff Fellow	Bg. 29, Rm. B12

Office Phone No.	FAX No.	Citizenship
402-1849		☒ U.S. ☐ Other:

Home Address
8507 Hazelwood Drive, Bethesda, MD 20814

Affiliation ☒ ICD *(specify ICD and applicable box below):* CBER/FDA

☐ GS ☐ CO ☐ Visiting Scientist ☐ Special Volunteer
☐ GM ☐ Visiting Fellow ☐ Howard Hughes Fellow ☒ Other (specify):
☐ SES ☐ Visiting Associate ☐ Guest Researcher Sr. Staff Fellow

☐ Non-ICD Affiliation *(specify):*

What specific contribution did she/he make to this work?
Protein sequencing, degenerate PCR strategy and cloning, identification in embryonic systems, localization in developing embryos and developmental assays.

Name	Degree	Social Security No.
Steve Chang	Medical Student	110-6433-17

Position Title	Office Address
Pre-IRTA Fellow	

Office Phone No.	FAX No.	Citizenship
(401) 435-6835		☐ U.S. ☒ Other: Taiwan

Home Address
Box G, Brown University, School of Medicine, Providence, RI

Affiliation ☒ ICD *(specify ICD and applicable box below):* NIDR

☐ GS ☐ CO ☐ Visiting Scientist ☐ Special
☐ GM ☐ Visiting Fellow ☐ Howard Hughes Fellow ☒ Other (specify):
☐ SES ☐ Visiting Associate ☐ Guest Researcher Pre- IRTA fellow

☐ Non-ICD Affiliation *(specify):*

What specific contribution did she/he make to this work?

cDNA cloning

Name	Degree	Social Security No.

Position Title	Office address

Office Phone No.	FAX No.	Citizenship
		☐ U.S. ☐ Other:

Home Address

Affiliation ☐ ICD *(specify ICD and applicable box below):*

☐ GS ☐ CO ☐ Visiting Scientist ☐ Special Volunteer
☐ GM ☐ Visiting Fellow ☐ Howard Hughes Fellow ☐ Other (specify):
☐ SES ☐ Visiting Associate ☐ Guest Researcher

☐ Non-ICD Affiliation 4 *(specify):*

What specific contribution did she/he make to this work?

Addendum to EIR Luyten/Moos

INTRODUCTION:

Because of the obvious importance of bone repair to orthopedics, reconstructive surgery, and dentistry, proteins that may facilitate this process have been sought for almost three decades. The starting point for these investigations was the observation that intramuscular implantation of demineralized bone incuces new bone formation. This cascade of biological events could be reproduced when 4 M guanidine-HCl extracts of demineralized bone were reconstituted with the residual bone powder. The ability to assay for cartilage and bone differentiation in ectopic non-skeletal sites in this manner permitted unambiguous identification of cartilage and bone inducing proteins and allowed osteogenic activity to be followed through several protein purification procedures. Three groups (Luyten et al., 1989; Wozney et al., 1988; Sampath et al., 1992) thus isolated material sufficiently homogeneous for peptide sequencing. The novel proteins discovered in this way were termed bone morphogenetic proteins (BMPs), and they were subsequently classified as members of the TGF-ß superfamily by virtue of amino acid sequence similarity. Screening of genomic and cDNA libraries led to the isolation of genes encoding BMP-2 through BMP-7. Many of these proteins, including homologs obtained from Drosophila, have been produced in recombinant expression systems and shown to initiate cartilage and bone formation *de novo*. Thus, the bone induction assay, while of crucial importance in the isolation of these activities, shows limited capacity to discriminate between the physiological roles of the different members of the BMP-family.This cartilage and bone inducing activity is remarkable in that the normal stages of endochondral bone formation occurring during ontogeny, including mesenchymal condensation, cartilage and bone marrow formation, and eventual mineralization to produce mature bone, are recapitulated in a normal adult animal.

Recent findings have indicated that the natural physiological functions of these proteins may be different and much more far-reaching than was originally anticipated. The observation that close homologs of these proteins exist in non-bony organisms such as Drosophila provided the first clue. Homologs of the BMP-2/BMP-4 subgroup exert profound effects on specification of the overall body plan, and organisms defective in these genes die during early

embryogenesis because of severe disturbances in anatomical pattern formation; this confirms the importance of these proteins in pattern formation. Accordingly, we feel that these proteins serve more than one function in mammals, and that many of them remain to be uncovered. Clearly, such efforts will widen the scope of potential therapeutic applications to include such areas as limb regeneration, hematopoiesis, and preservation or regeneration of muscle and nerve tissue in various traumatic and pathological contexts.

NOVEL FINDINGS:

(1) Ectopic implantation of partially extracted articular cartilage matrix from newborn calves induces direct bone formation without a cartilaginous intermediate stage and (2) subcutaneous implantation of bovine articular cartilage and fetal bovine epiphyseal cartilage extracts, when reconstituted with inactive bone residue, initiates *de novo* cartilage and bone formation in rats. We were therefore prompted to further characterize the cartilage derived molecules involved in the initiation of these inductive processes.

The characterization of the inductive activity has been approached by both protein purification and degenerate PCR strategies. Purification of cartilage extracts from bovine articular surfaces by 1.2 M guanidinium-HCl/CHAPS extraction, heparin affinity chromatography, molecular sieve chromatography and concanavalin A affinity chromatography, followed by micropreparative nondenaturing SDS-PAGE yielded a highly purified and bioactive (assay described in ref 1) fraction. The amino acid sequences of this protein fraction did not show significant homology to known proteins, including the previously described BMPs. These findings confirm the presence of a protein fraction in cartilage with cartilage and bone inductive activity, with characteristics resembling members of the BMP family, but apparently distinct in sequence from the known ones. Concurrently, oligonucleotide primers were designed from highly conserved regions of the mature portion of the BMPs. Using RT-PCR, several novel BMP-related fragments were identified. Isolation of the cDNA clones led to the discovery of at least two novel members of the morphogenetic protein family, probably representing a novel subfamily based on common sequence motifs. In contrast to the other members of the BMP family, their localization in human

tissues and development is restricted to chondrocytes, hypertrophic chondrocytes and progenitor cells in the bone forming regions of long bones. Parallel experiments in three lower vertebrate species (*G. Gallus*, *X. laevis* and *B. rerio*) confirmed the existence of these genes and suggested, by virtue of their high degree of amino acid sequence identity (>90%), that they are critical to the viability of all vertebrates. Recombinant protein from one of the novel genes was expressed in COS 1 cells and demonstrated *de novo* cartilage inducing activity *in vivo*.

CONCLUSION:

Each of the known BMPs examined so far initiates cartilage and bone differentiation when reconstituted with a pharmacologically inert carrier (e.g., collagen) in a rat subcutaneous implantation assay. As mentioned earlier, this suggests that this assay is not able to discriminate between closely related members of the family. This might reflect genomic compensatory mechanisms for potentially lethal damage in genes involved in skeletal development or body axis formation. However, the fact that BMP-4 null mice are embryonic lethal mutants, with severely deranged overall body anatomy, indicates that the situation is more complex. This is confirmed by the finding that the BMP homologs in Drosophila that are known to be involved in pattern formation (*decapentaplegic* and 60A) have the capacity to induce bone formation in mammals when produced in recombinant expression systems and bioassayed in the rat induction model. The reverse is also true: human BMP-4 is able to confer normal dorso-ventral patterning in Drosphila transformants defective in *dpp*. All these observations suggest that at least several BMPs may function as morphogens in development. The localization studies reported so far further support the contention that several BMPs have a more widespread role, such as signaling in epithelial-mesenchymal interactions in a number of tissues such as teeth, kidney, lung, heart and urinary bladder. The discovery of a novel subfamily of cartilage derived morphogenetic proteins with very restricted expression patterns might indicate the existence of morphogens with a primary physiological function in the induction and maintenance (i.e., balancing cartilage and bone formation at articular surfaces) of cartilaginous and bony tissues. Finally, this subfamily could include key molecules governing bone marrow differentiation.

Selected References:

Luyten, F.P., Cunningham, N.S., Ma, S., Muthukumaran, N., Hammonds, R.G., Nevins, W.B., Wood, W.I., and Reddi, A.H. (1989) Purification and partial amino acid sequence of osteogenin, a protein initiating bone differentiation. J. Biol. Chem. 264:13377-13380.

Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA (1988) Novel regulators of bone formation: molecular lones and activities. Science 242:1528.

Sampath TK, Coughlin JE, Whestone RM, Banach D, Corbett C, Özkaynak E, Oppermann H, Rueger D (1992) Bovine osteogenic protein is composed of dimers of OP-1 and BMP-2A, two members of the transforming growth factor-ß superfamily. J. Biol. Chem. 265:13198.

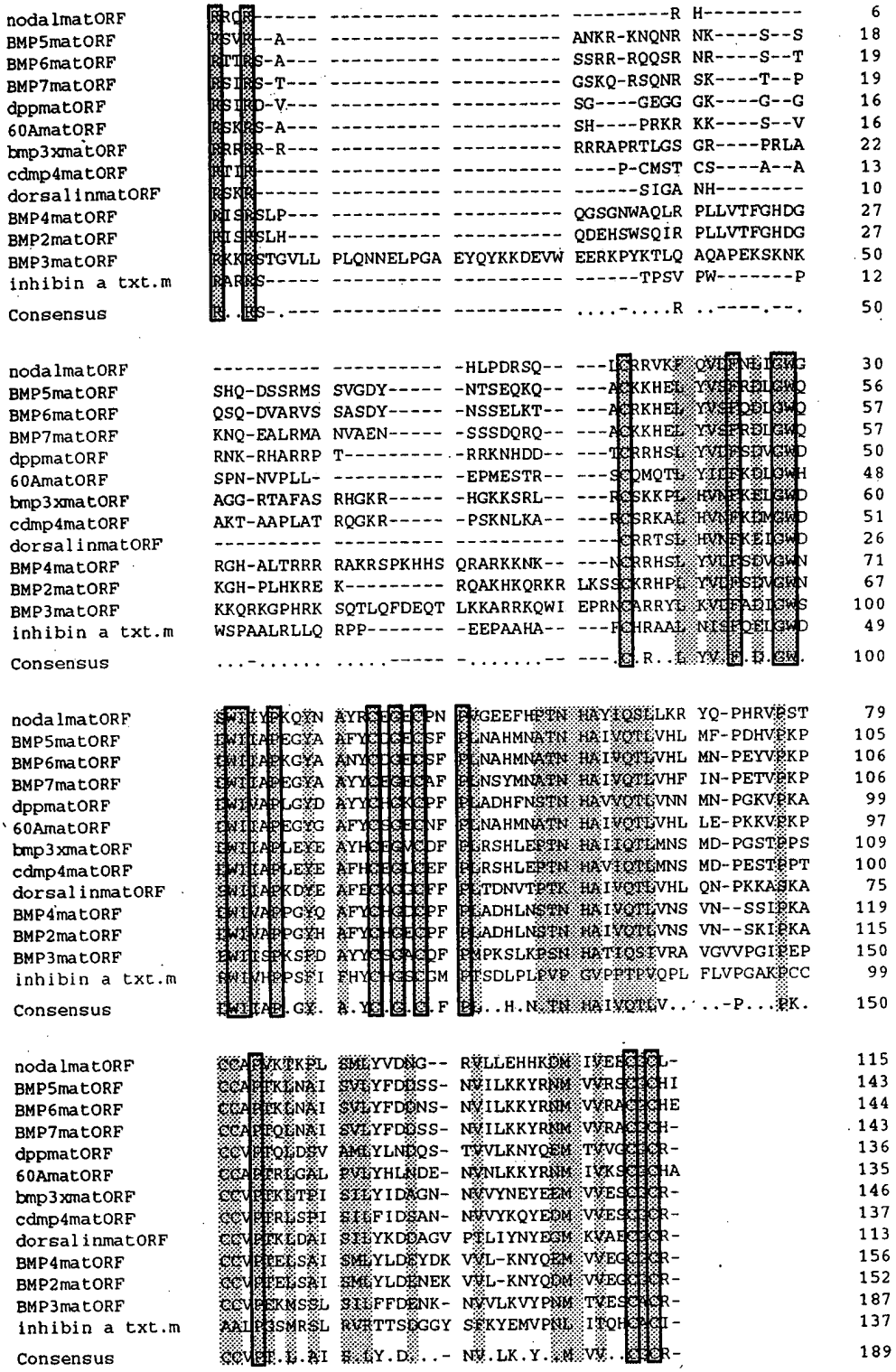
For review:

Reddi, A.H. (1992) Regulation of cartilage and bone differentiation by bone morphogenetic proteins. Curr. Opinion Cell Biol. 4:850-857.

Kingsley, D.M. (1994) What do BMPs do in mammals ? Clues from the mouse short-ear mutation. Trends in Genetics, 1, 16-21

Kingsley, D.M. (1994) The TGFß superfamily: new members, new receptors, and new genetic tests of function in different organisms. Genes and Development, 8: 133-146

Untitled-5 Formatted Alignment



consistent with a role for PKG in LTP, and suggest that under some circumstances cGMP analogues can also activate other mechanisms that may be involved in long-term depression.

The present results suggest that soluble guanylyl cyclase and PKG are involved in the induction of LTP and could serve as target proteins for retrograde messengers. Consistent with this idea, recent studies indicate that 8-Br-cGMP increases both the amplitude of evoked excitatory postsynaptic currents (e.p.s.cs) and the frequency of miniature e.p.s.cs in hippocampal cultures[21], suggesting that cGMP acts presynaptically. However, postsynaptic mechanisms may also be involved. cGMP and PKG have also been shown to produce activity-dependent long-lasting effects in cerebellum and sensory-motor cortex[22–24]. Because these effects are implicated in procedural learning[25,26], whereas LTP in hippocampus is thought to be involved in declarative learning[27], it is possible that these different types of learning at the behavioural level may involve, in part, similar underlying biochemical mechanisms. □

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ACKNOWLEDGEMENTS. We thank D. Sweatt for providing the protocol for the protein kinase assay, R. Y. Tsien, D. V. Madison and S. Siegelbaum for their comments on an earlier draft, S. Mack and C. Lam for preparing the figures and A. Krawetz and H. Ayers for typing the manuscript. This work was supported in part by grants from the National Institute on Aging, the National Institute of Mental Health and the Howard Hughes Medical Institute.

Limb alterations in *brachypodism* mice due to mutations in a new member of the TGFβ-superfamily

Elaine E. Storm*, Thanh V. Huynh†, Neal G. Copeland‡, Nancy A. Jenkins‡, David M. Kingsley*§ & Se-Jin Lee†

* Department of Developmental Biology, Beckman Center B300, Stanford University School of Medicine, Stanford, California 94305-5427, USA
† Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, 725 North Wolfe St, Baltimore, Maryland 21205, USA
‡ Mammalian Genetics Laboratory, ABL Basic Research Program, NCI Frederick Cancer Research and Development Center, PO Box B, Frederick, Maryland 21702-1201, USA

THE mutation *brachypodism* (*bp*) alters the length and number of bones in the limbs of mice but spares the axial skeleton[1,2]. It illustrates the importance of specific genes in controlling the morphogenesis of individual skeletal elements in the tetrapod limb[3,4]. We now report the isolation of three new members of the transforming growth factor-β (TGF-β) superfamily[5] (growth/differentiation factors (GDF) 5, 6 and 7) and show by mapping, expression patterns and sequencing that mutations in *Gdf5* are responsible for skeletal alterations in *bp* mice. GDF5 and the closely related GDF6 and GDF7 define a new subgroup of factors related to known bone- and cartilage-inducing molecules, the bone morphogenetic proteins (BMPs)[6]. Studies of *Bmp5* mutations in *short ear* mice have shown that at least one other BMP gene is also required for normal skeletal development[7]. The highly specific skeletal alterations in *bp* and *short ear* mice suggest that different members of the BMP family control the formation of different morphological features in the mammalian skeleton.

The *bp* mutation acts cell non-autonomously *in vitro* and *in vivo*, and may disrupt production of a signal that stimulates mesenchyme aggregation and chondrogenesis in limbs[8,9]. To test whether the skeletal alterations in brachypod mice could be due to mutations in a member of the BMP family of secreted signalling molecules we determined the mouse chromosome map locations of several BMP and BMP-related genes. The previously described *Op1*/(*Bmp7*)[10,11] and *Op2*/(*Bmp8*)[12] genes both map outside the candidate interval for the *bp* mutation, as do the genes for *Bmp2* -*Bmp6* (refs 7, 13).

Three new BMP-related factors were identified by degenerate polymerase chain reaction (PCR; Fig. 1) and designated GDF5, GDF6 and GDF7. Like other BMPs and TGF-β superfamily members, each of the new factors contains a putative polybasic proteolytic processing site followed by a carboxy-terminal region containing seven conserved cysteine residues[5]. Previously characterized BMPs fall into three homology groups[6,12]: the BMP2/BMP4 subfamily; the Vgr1(BMP6)/OP1(BMP7)/BMP5/OP2(BMP8) subfamily; and BMP3. Molecules within each group share 74–92% identity in the amino-acid sequence of the mature C-terminal signalling region. Members of different subfamilies share 40–60% identity. With the exception of a 26-amino-acid glycine-rich insert present in GDF7, GDF5, GDF6 and GDF7 share 80–86% identity with each other in the mature C-terminal region, and 56–57%, 50–54% and 46–47% identity respectively with members of the three BMP subfamilies mentioned. Lower identity scores were seen with other TGF-β superfamily members. These comparisons suggest that GDF5, GDF6 and GDF7 genes define a new subgroup of BMP-related factors. Chromosome mapping has shown that the *Gdf5* locus maps between the *agouti* and *Src* loci on chromosome 2 (Fig. 2). The *bp* locus has been mapped to the same region[14], identifying *Gdf5* as a candidate for the gene defective in brachypod mutants. *Gdf6* and *Gdf7* map to the proximal ends of chromosomes 4 and 12 and are not obvious candidates for other known mouse mutations (data not shown).

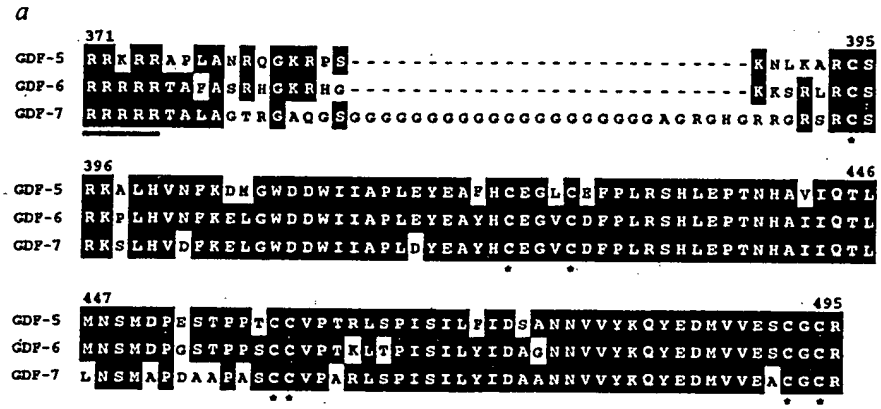
To test whether GDF5 is the normal product of the *bp* locus, we first isolated a full-length complementary DNA clone for the wild-type transcript. A probe from the mature region of GDF5 hybridized to a single 2.5-kilobase (kb) transcript in embryonic RNA, peaking at day 12.5 of development (data not shown). The complete sequence of a 2,329-base-pair (bp) cDNA clone of the embryonic transcript is shown in Fig. 1*b*. The wild-type sequence contains a single large open reading frame of 495 amino acids.

We next looked for alterations of *Gdf5* sequences in three independent *bp* mutations. Two of the mutations arose on the

§ To whom correspondence should be addressed.

A/J and BALB/cJ inbred mouse strains, which were included in all experiments as parental controls (Fig. 3 legend). Southern blot analysis did not detect major disruptions of *Gdf5* genomic sequences in any of the mutations; we therefore amplified coding sequences from RNA and analysed them by DNA sequencing. All three mutant alleles contained frameshift mutations in the *Gdf5* open reading frame (Fig. 3). In *bp/bp* mice, bases 477–483 of the wild-type sequence are deleted, followed by an inversion of bases 484–496. The resulting frameshift creates a translational stop codon 62 amino acids later. In bp^{3J}/bp^{3J} mice, a CG dinucleotide in the wild-type sequence is replaced by a single T at position 876. This frameshift mutation produces a translational stop at the next codon. In bp^{J}/bp^{J} mice, a stretch of three Gs in the wild-type sequence is replaced by four Gs in the mutant (positions 1,444–1,448). This change produces a stop codon 41 amino acids later. Neither sequence alteration found in the bp^{J} and bp^{3J} mutants was present in the A/J or BALB/cJ parental control strains, strongly arguing that each was the result of a new mutation at the *bp* locus.

Active signalling molecules in the TGF-β superfamily are formed from the C-terminus of a larger precursor protein[5]. The frameshift mutations in *bp*, bp^{J} and bp^{3J} all occur before the mature signalling portion of GDF5 and should represent functional null mutations. To compare the skeletal phenotypes of the different alleles, we prepared skeletons from mice homozygous for the bp^{J} and bp^{3J} mutations. As reported for the *bp* mutation on an outbred background[1,2], the new mutations on coisogenic backgrounds produce specific defects in limb morphology (Fig. 4*a*, *b*, and other data not shown). The long bones of the limb are slightly shorter, and the feet are much



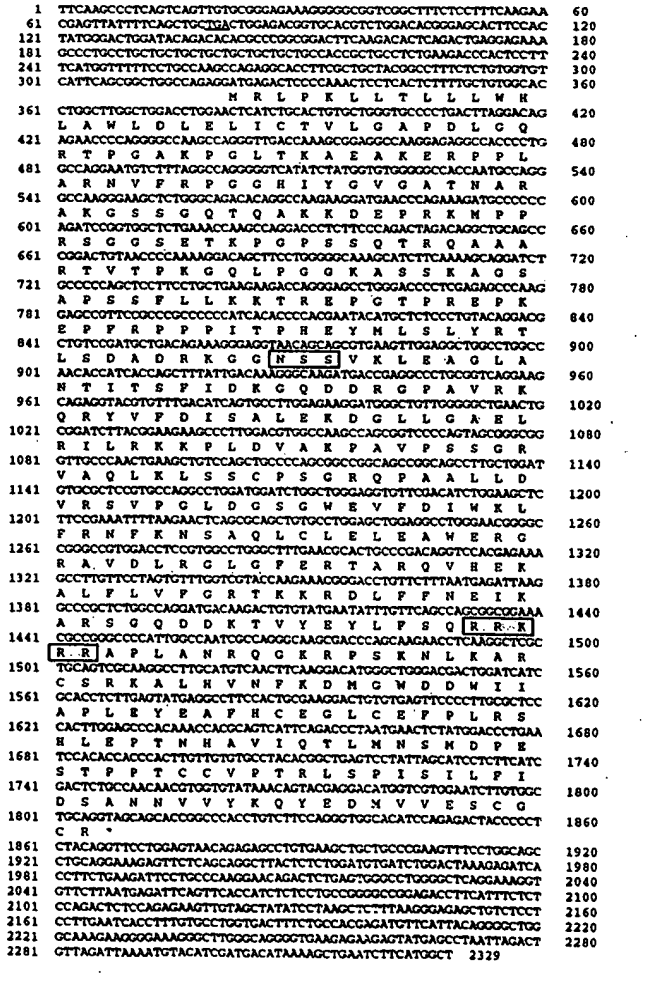


FIG. 1 Sequences of three new members of the TFG-β superfamily. *a*, Partial amino-acid sequences of GDF5, GDF6 and GDF7 are aligned beginning at their predicted protease recognition sites (underlined). Dashes denote gaps introduced to maximize the alignment. Numbers indicate amino-acid positions in GDF5. The seven conserved cysteine residues are indicated (*). Identical amino acids are shaded. *b*, Nucleotide sequence of a full-length *Gdf5* cDNA clone. The predicted GDF5 product is a 495-amino-acid, 54.9K protein with a putative proteolytic processing site (shaded box) preceding a 120-amino-acid, 13.6K mature C-terminal polypeptide. A single *N*-linked glycosylation site is located in the pro-region (open box). An in-frame termination codon upstream of the putative ATG initiation codon is underlined. The poly(A) tail is not shown.

METHODS. The following oligonucleotide primers were used to screen mouse genomic DNA for new members of the TFG-β superfamily by PCR. Corresponding amino-acid sequences are shown in brackets. SJL136: CCGGAATTCGG(G/A/T/C)TGGGA(G/A)(A/C)G(G/A/T/C)TGG(G/A)T(G/A/T/C)(G/A)T [GWE(R/S)W(V/I/M)(V/I/M)]; SJL121: CCGGAATTC(G/A)-CAICC(G/A)CA(T/C)TC(G/A)TCIACIACCAT(G/A)TC(T/C)TC(G/A)TA [reverse complement: YEDMVVDECGC]; SJL141: CCGGAATTCGGITGG(G/C/A)A-(G/A/T/C)(A/G)A(T/C)TGG(A/G)TI(A/G)TI(T/G)CICC [GW(H/Q/N/K/D/E)-(D/N)W(V/I/M)(V/I/M)(A/S)P]; SJL145: CCGGAATTC(G/A)CAI(G/C)C(G/A)CAIG(C/A)(G/A/T/C)TCIACI(G/A)(T/C)CAT [reverse complement: M(V/I/M/T/A)V(D/E)(A/S)C(G/A)C]; and SJL146: CCGGAATTC(G/A)CAI(G/C)C-(G/A)CAIG(C/A)(G/A/T/C)C(G/T)IACI(G/A)(T/C)CAT [reverse complement: M(V/I/M/T/A)V(R/S)(A/S)C(G/A)C]. PCR was carried out at 94 °C for 1 min, 50 °C for 2 min, and 72 °C for 2 or 3.5 min for 40 cycles with 2 μg CD-1 mouse genomic DNA as template. Initial GDF5, GDF6 and GDF7 sequences were identified using primers SJL136 and SJL121, SJL141 and SJL145, and SJL141 and SJL146, respectively. The GDF6 and GDF7 sequences were used to screen a mouse BALB/c genomic library from A. Lanahan. GDF5 sequences were used to screen a cDNA library constructed in the λZAPII vector (Stratagene) from day 12.5 post-coitum (p.c.) CD-1 mouse embryo poly(A)$^+$ RNA. PCR products, genomic clones and the longest cDNA clone were sequenced as described[20].

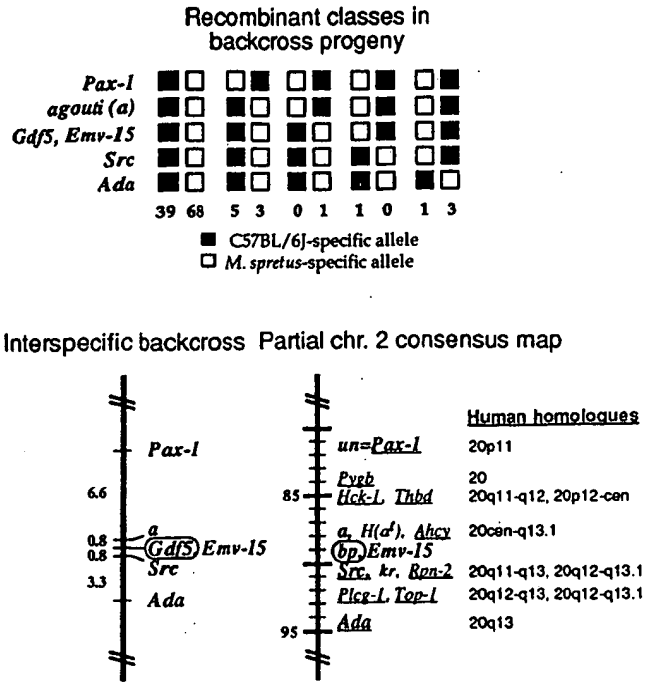


FIG. 2 *Gdf5* maps in the *bp* region of mouse chromosome 2. Top, recombination mapping places *Gdf5* between the *agouti* and *Src* loci on chromosome 2, closely linked to *Emv-15;* bottom, comparison of *Gdf5* map position with a partial consensus linkage map of distal mouse chromosome 2 (ref. 14). The *bp* locus has also been placed between *agouti* and *Src*, closely linked to *Emv-15*. Several underlined loci both proximal and distal to this region have been mapped to chromosome 20 in humans (map locations shown on the right)[14].
METHODS. DNA was prepared from progeny of an interspecific backcross between (C57BL6J × *M. spretus*) F_1 females and C57BL/6J males, digested with *Hin*cII, and analysed by Southern blot hybridization with a *Gdf5* cDNA probe (nucleotides 1,997–2,329). This probe hybridized to a 3.7-kb *Hin*cII fragment in C57BL/6J DNA, a 2.6-kb fragment in *M. spretus* DNA, and to one or both fragments in backcross progeny. The inheritance of this restriction-fragment length polymorphism in 121 backcross progeny was compared with the inheritance patterns of over 2,000 other markers previously typed on the same animals[21], including *Pax-1, a, Emv-15, Src* and *Ada*[22,23]. Columns of black and white boxes represent chromosome types inherited from the F_1 hybrid parent. Numbers beneath the columns represent the number of backcross progeny observed with each chromosome type.

shorter than controls. The reduction in length of the feet is largely the result of altered patterning of segments in the digits. In place of the proximal and medial phalanges is a single bone that has been described previously as resulting from a fusion of the proximal and medial phalangeal condensations (Fig. 4*b*)[2]. Additional defects in the length of the metacarpals and metatarsals and slight disorganization of the carpals and tarsals are also present. In contrast, the axial skeleton is largely unaffected by the mutations.

Previous studies have shown that defects in brachypod mice are first detectable around day 12 of gestation[2], when mesenchyme first aggregates into outlines of future digit elements. Digit condensations in brachypod mice are thin, malformed, and slow to initiate chondrogenesis. In addition, mesenchyme from brachypod mice shows a reduced ability to form aggregates and cartilaginous nodules *in vitro*[9,15]. To determine the sites of *Gdf5* expression in wild-type embryos, serial sections of day-12.5 embryos were analysed by *in situ* hybridization. *Gdf5* transcripts were detected in the limbs in distal precartilaginous mesenchymal condensations and in the perichondrium of more proximal skeletal structures (Fig. 4*c–f*). No other major sites of hybridization were detected in axial skeletal structures or soft tissues.

The sequencing studies reported here provide strong genetic evidence that the skeletal alterations in *bp* mice are the result of mutations in *Gdf5*. This conclusion is further supported by the map location of *Gdf5*, its homology to BMPs, its specific expression in the mesenchymal condensations that give rise to the limb

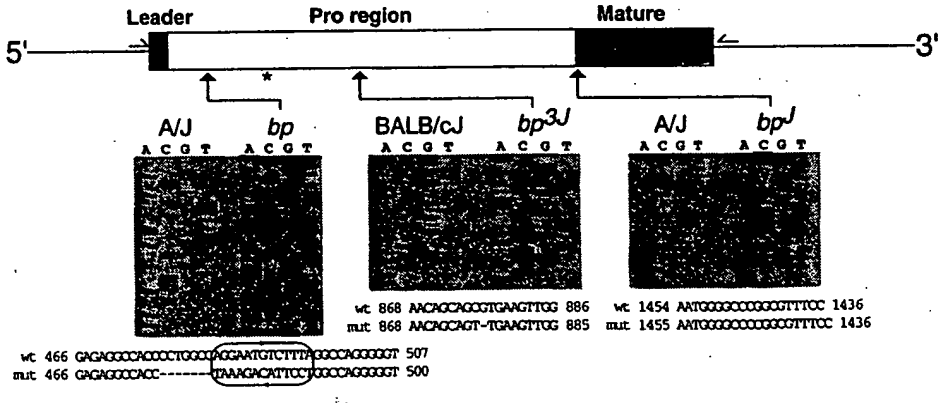


FIG. 3 Three independent *bp* mutations disrupt *Gdf5* coding sequences. Top, schematic diagram of the *Gdf5* cDNA showing positions of sequence alterations in *bp* alleles. All three mutations result in frameshifts and premature translational termination in the GDF5 open reading frame. The *bp* mutation occurred spontaneously on an outbred mouse strain in 1952[1]. The bp^J and bp^{3J} mutations occurred spontaneously in the highly inbred A/J and BALB/cJ wild-type strains in 1975 and 1993, respectively[24]. Position 613 (asterisk) is polymorphic in different strains, resulting in a serine residue in CD-1, BALB/cJ and bp^{3J} mice, and a proline residue in the A/J, bp^J and *bp* strains. Bottom, details of frameshift mutations. Wild-type and mutant sequence information is presented beneath autoradiograms demonstrating the sequence changes. The reverse complement of bases 1,436–1,454 is shown.
METHODS. *Gdf5* coding sequences were amplified from control and mutant RNA using reverse transcription and PCR. RNA was isolated from brains using RNAzol (CINNA-BIOTECX). First-strand reverse transcription reactions used primers located from 1,869 to 1,850 or 1,575 to 1,554 in the wild-type *Gdf5* cDNA sequence. cDNA reactions were then amplified with primer pairs 1,306–1,322 and 1,869–1,850, or 272–289 and 1,575–1,554. Second-round amplifications used primers 1,364–1,384 and 1,869–1,850, or 290–308 and 1,497–1,480. Amplified PCR products were cut from Sea Plaque (FMC) agarose and sequenced directly using the Sequenase Version 2.0 sequencing kit (USB). Sequencing primers correspond to positions 294–311, 767–783, 846–829, 1,364–1,384, 1,497–1,480 and 1,869–1,850 in the cDNA. Arrows bracket the region completely sequenced in two control strains and the three mutant alleles (position 320 to 1,814). Each frameshift mutation was confirmed on both strands and in at least two independent reverse-transcription PCR reactions.

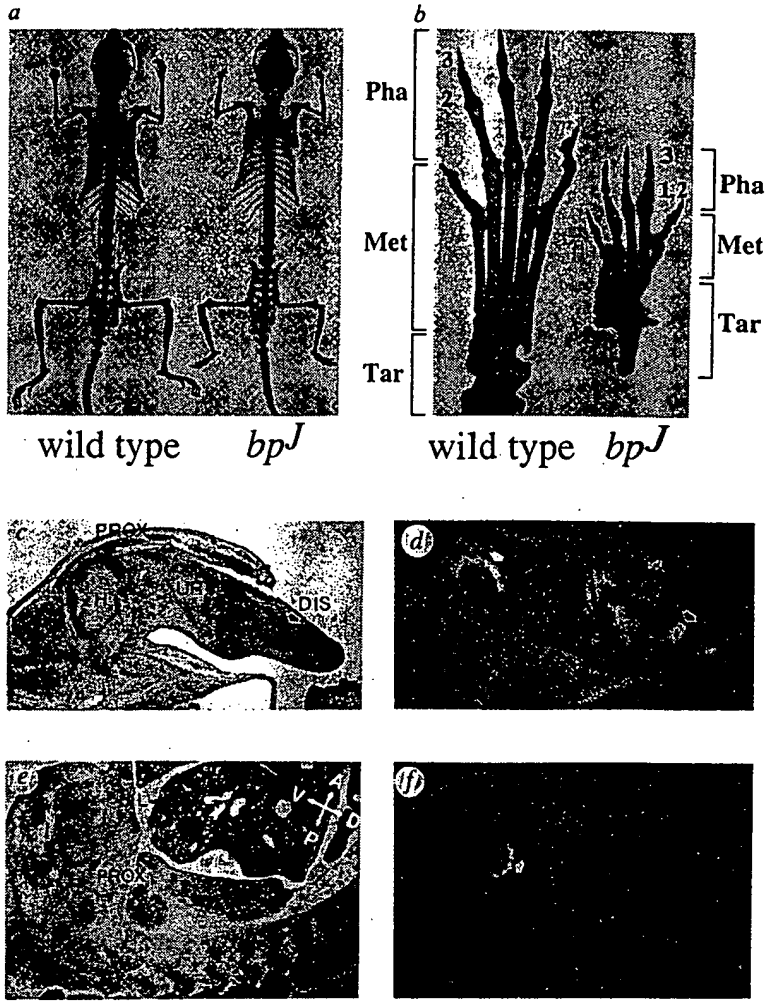


FIG. 4 Skeletal phenotype of bp^J mutant and *Gdf5* expression in the developing limb. *a*, Alizarin red skeletal preparations from a representative bp^J homozygote and an age-matched wild-type animal from the parental A/J inbred strain. The long bones are reduced and the fibula is shortened proximally. The bones of the axial skeleton are unaffected. *b*, close-up view of right hind feet. The most prominent alteration is a change in the number of the phalanges in bp^J homozygotes. In addition, metatarsals are reduced in length and the tarsals are irregular in shape. *c–f*, Expression of *Gdf5* in limb mesenchyme of day-12.5 p.c. mouse embryos. Bright-field (*c*, *e*) and dark-field (*d*, *f*) photomicrographs of transverse (*c*, *d*) and sagittal (*e*, *f*) sections, showing views through forelimb and posterior end of embryo, respectively, after hybridization with ^{35}S-labelled *Gdf5* antisense probe. Serial sections revealed hybridization to be localized to proximal (PROX, filled arrows) and distal (DIS, open arrows) mesenchyme in the forelimb (*c*, *d*) and hindlimb (*e*, *f*). Anterior–posterior (A–P) and dorsal–ventral (D–V) embryonic axes are indicated (*e*). UR, H and DB indicate primordia of ulna and radius, humerus, and digital bones; L indicates liver. *Gdf5* sense control probes produced no signals on adjacent sections (data not shown).

METHODS. Skeletons of mice were prepared in 1% (w/v) potassium hydroxide and stained using Alizarin red[25]. Day-12.5 p.c. female mouse embryos were fixed, embedded in paraffin, sectioned and hybridized as described[26] to sense or antisense RNA probes (4×10^5 c.p.m. μl^{-1}) transcribed from templates containing nucleotides 308–1,446 of the *Gdf5* cDNA clone. Slides were developed after a 4–6-week exposure time to Kodak NTB3 emulsion and were stained with haematoxylin and eosin.

skeleton, and previous experiments showing that the *bp* mutation acts cell non-autonomously in cultured cells and chimaeric mice[8,9]. The remarkable ability of BMPs to induce ectopic bone and cartilage[6] and the defects in skeletal structures caused by *Bmp5* mutations in *short ear* mice[7] has suggested that BMPs are the normal signals used to initiate bone and cartilage formation during embryonic development[16]. Our results strongly support this model and provide evidence that BMP-related genes are also required for skeletal patterning in the vertebrate limb. Strikingly, although the *brachypodism* and *short ear* mutations both disrupt the condensation of mesenchyme cells into outlines of particular skeletal elements, they do so in completely different regions of the embryo: *short ear* null mutations alter the size and shape of ears, sternum, ribs and vertebral processes, but do not alter limb bone lengths or digit morphology, whereas *bp* null mutations alter bone lengths and the number of segments in the digits of all four limbs, but do not affect ear, sternum, rib or vertebral morphology. The skeleton of higher animals thus appears to be a mosaic structure that is built from the composite patterns of activity of different BMP-like proteins. Changes in the activity of particular BMP family members may provide a general mechanism for altering the number and morphology of individual skeletal elements during development and evolution.

Although *Gdf5* transcripts are present in a variety of non-skeletal tissues in adult mice (including uterus, placenta, oviduct, brain, thymus, heart, lung, kidney and adrenal gland; data not shown), brachypod mice are fertile and do not show obvious behavioural abnormalities or striking morphological changes in soft tissues. More detailed studies will be required to determine whether subtle defects are present, or whether related family members can compensate for GDF5 deficiencies in soft tissues. A number of human brachydactyly syndromes are known that also disrupt the size or number of phalanges and limb bones without producing obvious abnormalities in other organ systems[17–19]. Direct analysis of *Gdf5* sequences or linkage studies with chromosome 20 markers (Fig. 2) can now be used to test whether any of these human syndromes are also due to *Gdf5* mutations. □

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Ligand for FLT3/FLK2 receptor tyrosine kinase regulates growth of haematopoietic stem cells and is encoded by variant RNAs

C. Hannum, J. Culpepper, D. Campbell, T. McClanahan, S. Zurawski, J. F. Bazan, R. Kastelein, S. Hudak, J. Wagner, J. Mattson, J. Luh, G. Duda, N. Martina, D. Peterson, S. Menon, A. Shanafelt, M. Muench, G. Kelner, R. Namikawa, D. Rennick, M.-G. Roncarolo, A. Zlotnik, O. Rosnet*, P. Dubreuil*, D. Birnbaum* & F. Lee

DNAX Research Institute of Molecular and Cellular Biology, 901 California Avenue, Palo Alto, California 94304-1104, USA
*U119 INSERM, 27 Boulevard Lei Roure, 13009 Marseille, France

THE FLT3/FLK2 receptor tyrosine kinase is closely related to two receptors, c-Kit and c-Fms, which function with their respective ligands, Kit ligand and macrophage colony-stimulating factor to control differentiation of haematopoietic and non-haematopoietic cells[1–5]. FLT3/FLK2 is thought to be present on haematopoietic stem cells and found in brain, placenta and testis[3–5]. We have purified to homogeneity and partially sequenced a soluble form of the FLT3/FLK2 ligand produced by mouse thymic stromal cells. We isolated several mouse and human complementary DNAs that

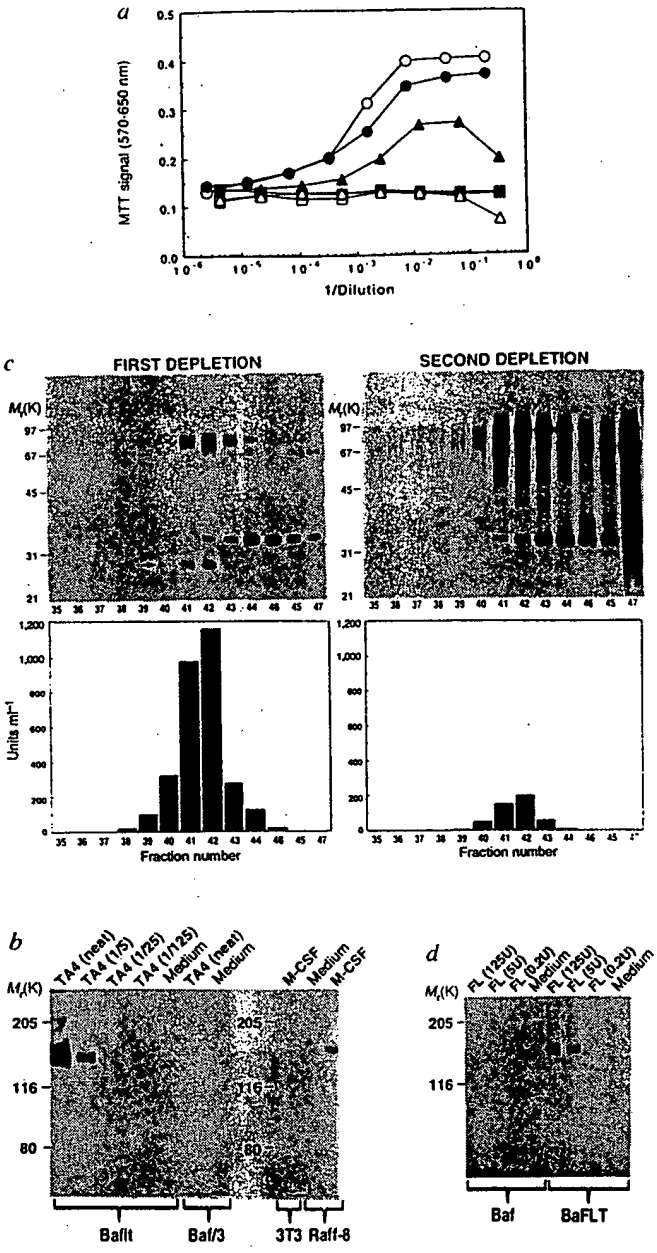

FIG. 1 FL assays and purification *a*, Survival assay (measured by 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide conversion) in which concentrated TA4-conditioned medium (neat, 100×) is titrated onto factor-dependent Ba/F3 (–△–) and Baflt (–▲–) cells. Also shown is the Ba/F3 (–○–) and Baflt (–●–) response to IL-3 and the Ba/F3 (–□–) and Baflt (–■–) response to medium alone. *b*, Autophosphorylation assay (measured by the appearance of an immunoprecipitated FLT3 band on an antiphosphotyrosine western blot) in which TA4-conditioned medium is titrated onto Ba/F3 and Baflt cells. As a positive control, the tyrosine-phosphorylated receptor was precipitated from M-CSF-stimulated Raff-8 cells (Rat-2 fibroblasts transfected with a chimaeric receptor composed of the ligand binding domain of c-Fms and the intracellular domain of FLT3/FLK2). *c*, Analysis of fractions from reversed-phase HPLC of affinity-purified FL from the consecutive first (left) and second (right) affinity depletions. Top, silver-stained SDS–PAGE on reduced samples of each fraction in the region of FL activity. FL is the ~30K band whose appearance coincides with biological activity. Lower, FL activity in each fraction as measured using the Baflt-Ba/F3 survival assay. In this case fractions 40–43 from the first depletion were then combined and run on preparative SDS–PAGE (not shown) to purify the FL to homogeneity for sequence analysis. The crude TA4-conditioned medium contains about 0.05 ng FL per ml, and affinity chromatography plus RP-HPLC produces biologically active FL that is purified >300,000-fold. *d*, Autophosphorylation assay using highly purified natural FL.

METHODS. To measure FL biological activity, MTT colorimetric assays[13] were performed in parallel on Ba/F3 and Baflt cells using 10^4 cells per well. The FL-specific dose response was calculated by subtracting the Ba/F3 titration signal from the corresponding Baflt titration signals. Unit of FL activity, amount of active material producing 50% maximal stimulation. For receptor phosphorylation assays, 3×10^6 Baflt or Ba/F3 cells were stimulated with ligand for 5 min. Cell lysates were prepared and immunoprecipitated with an antiserum against the kinase-insert domain of FLT3/FLK2[14]. This antiserum was also used to detect the chimaeric c-Fms/FLT3 receptor on Raff-8 cells. Immunoprecipitates were run on SDS–PAGE and western blots probed with an antiphosphotyrosine antibody (4G10). Typically >90% of the FL in 200 l of TA4-conditioned medium was adsorbed with 5 ml FLT3/FLK2 affinity beads, eluted with 0.1M glycine, pH 2.5, and chromatographed on a 4.6 × 200 mm Poros R/H (PerSeptive) reversed-phase column with a linear 28% to 40% CH_3CN gradient (into H_2O) with 0.1% TFA. Active fractions were combined and run in a single lane of an SDS–PAGE minigel. The FL band was then either blotted to PVDF membrane for direct N-terminal sequencing or excised and digested with endoproteinases (trypsin, chymotrypsin, or AspN) for sequence determination of peptides purified by microbore RP-HPLC. Sequencing was done with Applied Biosystems 476A and 477A gas phase sequencers.

The BMP proteins in bone formation and repair

VICKI ROSEN AND R. SCOTT THIES

From recent advances in the fields of bone biology and pattern formation, the first clues to our understanding of embryonic skeletal development are beginning to emerge. This complex process involves an integration of spatial patterning and the differentiation of specialized cells that make up bone and cartilage. The result is a scale model of the mature skeleton which is able to grow in size to fit the adult body plan. In the mature animal, bone repair after injury appears to be similar to bone formation in the embryo, suggesting that analogous mechanisms for the control of bone formation may exist in the adult and embryonic skeletons.

In the vertebrate embryo, the first stage of bone formation may involve either the establishment of a cartilaginous intermediate (endochondral bone formation), or direct conversion of mesenchymal cells into osteoprogenitors (intramembranous bone formation) or, in some instances, a combination of these two processes. Bones of the limbs, vertebral column, pelvis and the base of the skull form by the endochondral pathway[1], and require the production of a cartilaginous prototype. This process has been extensively studied histologically, but the absence of specific markers for mesenchymal stem cells and their progeny has limited our understanding to a descriptive level.

The transformation from mesenchyme to cartilage begins when histologically undifferentiated mesenchymal cells migrate to precise locations in the embryo[2]. These cells condense, growing into tightly packed amalgams which secrete large amounts of extracellular matrix components that are characteristic of cartilage, notably collagen type II and cartilage-specific proteoglycans (Fig. 1, top left). Layers of fibroblast-like cells form around each cartilage nodule, creating a boundary between the developing cartilage and the surrounding tissue. This perichondrium becomes infiltrated by capillaries on its outer surface, providing vascular support for continued growth of the cartilage nodule[3] (Fig. 1, top right).

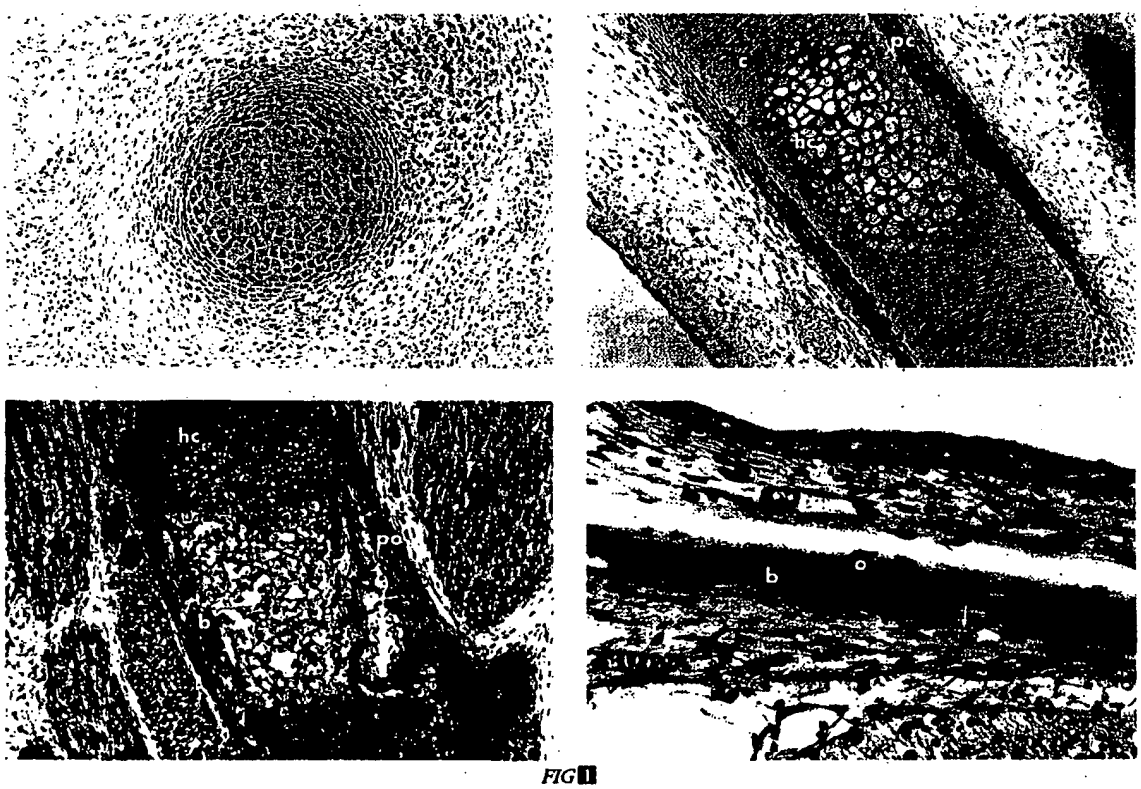

FIG 1

Photomicrographs of bone formation in the mouse embryo. Top left: Cross-section through forelimb of 13.5 d.p.c. (days *post coitum*) mouse embryo. The area of chondrogenesis shown is at the level of the shoulder. The section has been stained for alkaline phosphatase (red) and counterstained with toluidine blue. c, cartilage; pc, perichondrium. Top right: Longitudinal section through forelimb of 16 d.p.c. mouse embryo. The area shown is at the level of the digits. Staining as above. c, cartilage; pc, perichondrium; hc, hypertrophic cartilage. Arrows point to sites of vascular invasion. Bottom left: Longitudinal section through forelimb of 16 d.p.c. mouse embryo. The area shown is at the level of the humerus. The section has been stained with toluidine blue. hc, hypertrophic cartilage; po, periosteum; b, bone. Arrows point to sites of vascularization. Bottom right: Longitudinal section through calvaria (developing skull) of 16 d.p.c. mouse embryo. The section has been stained with toluidine blue. b, bone; o, osteoblasts. Arrow points to site of vascular invasion. Photos courtesy of J. Capparella.

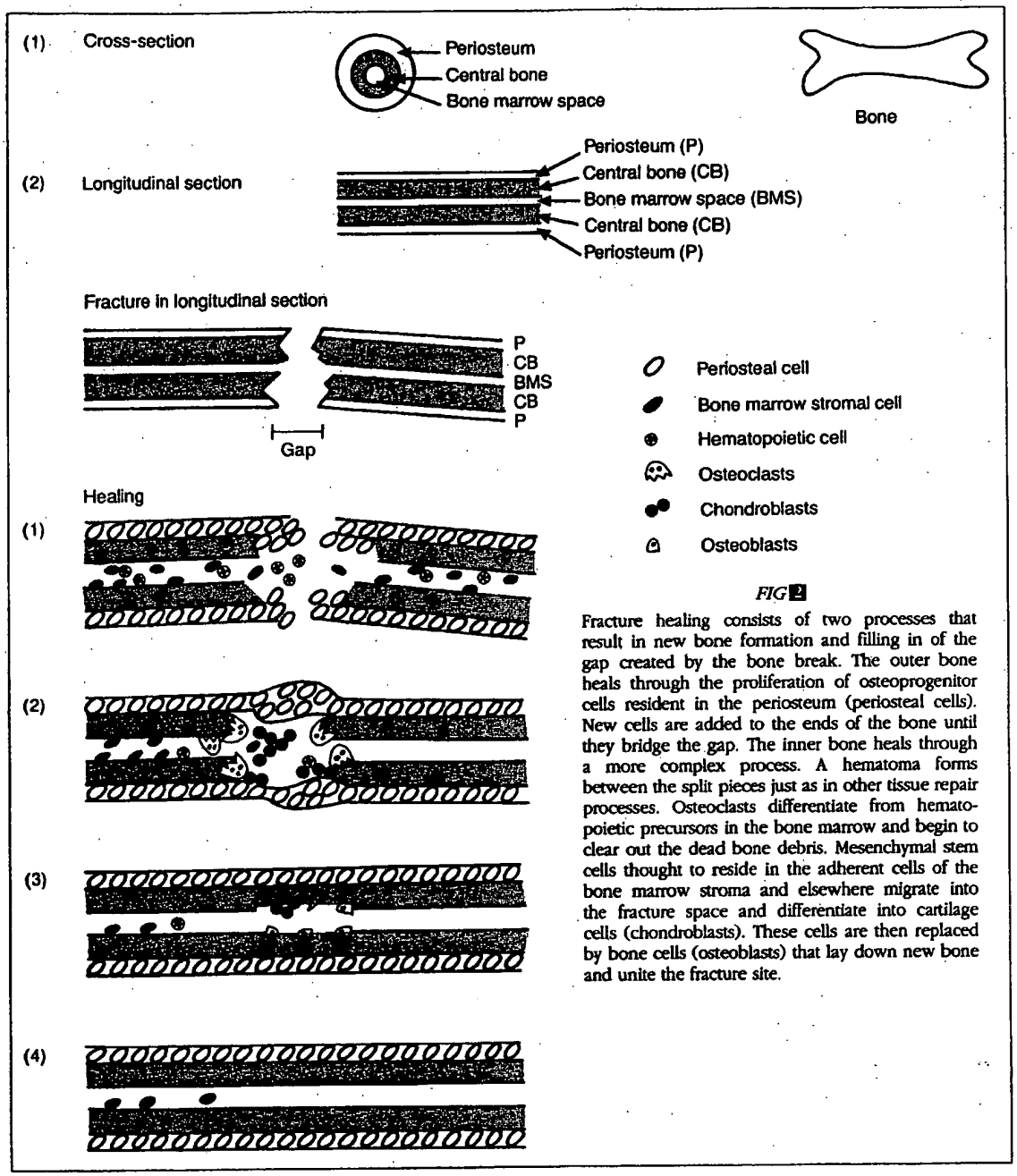

FIG 2

Fracture healing consists of two processes that result in new bone formation and filling in of the gap created by the bone break. The outer bone heals through the proliferation of osteoprogenitor cells resident in the periosteum (periosteal cells). New cells are added to the ends of the bone until they bridge the gap. The inner bone heals through a more complex process. A hematoma forms between the split pieces just as in other tissue repair processes. Osteoclasts differentiate from hematopoietic precursors in the bone marrow and begin to clear out the dead bone debris. Mesenchymal stem cells thought to reside in the adherent cells of the bone marrow stroma and elsewhere migrate into the fracture space and differentiate into cartilage cells (chondroblasts). These cells are then replaced by bone cells (osteoblasts) that lay down new bone and unite the fracture site.

As the cartilage cells in the middle of the anlage hypertrophy, their matrix becomes mineralized, presumably limiting the availability of nutrients and gas exchange, and the hypertrophic chondrocytes die. Subsequently, phagocytic cells, entering the developing bone through newly formed blood vessels, remove the dead cartilage and create a cavity that becomes occupied by osteoprogenitor cells. The source of these bone-forming cells is unknown. They may differentiate from progenitors present in the fibroblast layers of the perichondrium, or perhaps from the vascular system (Fig. 1, bottom left).

As osteoblasts differentiate from progenitors, they synthesize and deposit bone-specific matrix proteins[4]. Infiltrating vascular components form an intricate series of channels throughout the bone, providing nutrients and allowing gas exchange. The final result of this highly complex process is a bone surrounded by periosteum and containing a central marrow cavity. The newly formed bone may grow in size as existing osteoprogenitor cells mature, without having to return to the formation of a cartilaginous intermediate.

Bones of the craniofacial skeleton – the maxilla, mandible and skull – undergo a slightly different

developmental progression known as intramembranous bone formation. During this process, progenitor cells from the neural crest and other areas migrate and condense at appropriate sites in the embryo[5]. In intramembranous bone formation, the condensing cells differentiate directly into osteoblasts, as judged by morphological and biochemical parameters (Fig. 1, bottom right). Thus, unlike endochondral bone formation, this process does not appear to involve an intermediate cartilaginous state. However, recent evidence suggests that the osteogenic cells of the cranial mesenchyme transiently express cartilage-specific collagens at fairly high levels before they differentiate into osteoblasts[6], making the distinction between intramembranous and endochondral bone formation less clear cut.

In cases of fracture repair, osteogenesis closely resembles the stages of intramembranous and endochondral bone formation seen in embryogenesis. As illustrated in Fig. 2, repair proceeds along two fronts. The fibroblast layer of the periosteum begins a sustained period of cell division to increase the osteoprogenitor population. These progenitor cells then differentiate into mature osteoblasts, which produce enough new bone matrix to close the gap between the cortical surfaces[7]. This process is analogous to intramembranous bone formation, and no cartilaginous intermediate is evident. The central core of bone is repaired in a process resembling endochondral bone formation. Mesenchymal cells brought into the site differentiate into chondroblasts, give rise to a calcified cartilage matrix and subsequently to new bone and bone marrow. Since these events so closely resemble those seen in the embryo, it is possible that the same regulatory signals and responsive cell types are required for fracture repair and embryonic skeleton formation. This possibility will remain untested until we learn more about the cell populations involved.

Discovery of osteoinductive proteins

Osteoinductive factors were discovered when it was noticed that pieces of demineralized bone, or extracts isolated from osteosarcomas and certain epithelia, could direct new cartilage and bone formation when placed subcutaneously or intramuscularly in rats[8–10]. This activity was named bone morphogenetic protein (BMP) by Marshall Urist in 1965, but the proteins responsible for bone induction remained unknown until the cloning[11] of human proteins BMP-2–4 in 1988. After BMP is implanted ectopically, mesenchymal cells appear at the site, where they proliferate and differentiate into chondroblasts. The cartilage formed then hypertrophies, calcifies and is eventually replaced by bone, complete with a functional marrow cavity[12]. This sequence of events closely resembles endochondral bone formation as it occurs during embryogenesis and in fracture healing, and suggests that the proteins responsible for bone induction in this assay may be the natural bone inductive factors.

At present, seven different proteins in the BMP fraction of bovine bone have been described. Six of these appear to be closely related, on the basis of sequence homology (Fig. 3a). Molecular cloning of the

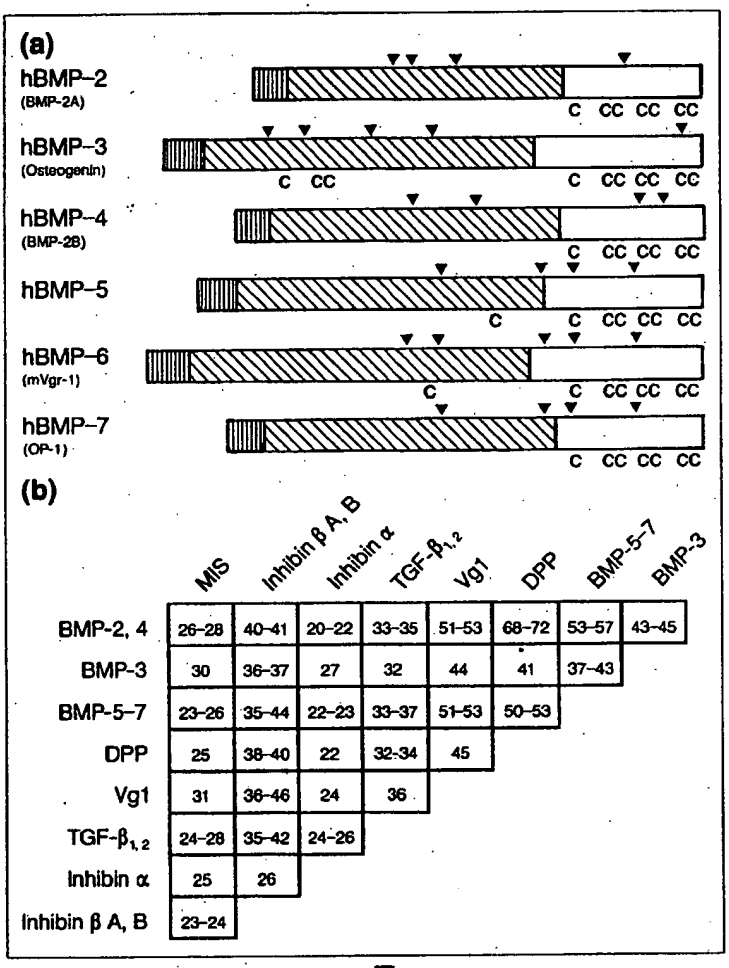

FIG 3

Comparisons of the BMP proteins and the homologies of proteins in the TGF-β family. (a) Comparison of the BMPs before they are processed for secretion. The striped areas represent the hydrophobic leader sequences of each BMP precursor. The propeptide portion of each BMP is indicated by the hatched region and the mature peptide sequences are shown by the open rectangles. The active portion of each BMP is the carboxy-terminal domain, which contains seven conserved cysteines (C). All the BMPs are glycosylated and potential *N*-linked glycosylation sites are represented by filled triangles. OP-1, osteogenic protein 1; h, human; m, mouse. (b) Comparison of the amino acid sequences of the mature regions of the TGF-β family members. BMP sequences used for comparison are the human proteins, as are the sequences of Müllerian inhibitory substance (MIS), the inhibins, and the transforming growth factor β (TGF-β) molecules. DPP (decapentaplegic) is the *Drosophila* protein, and Vg1 is the *Xenopus* mRNA.

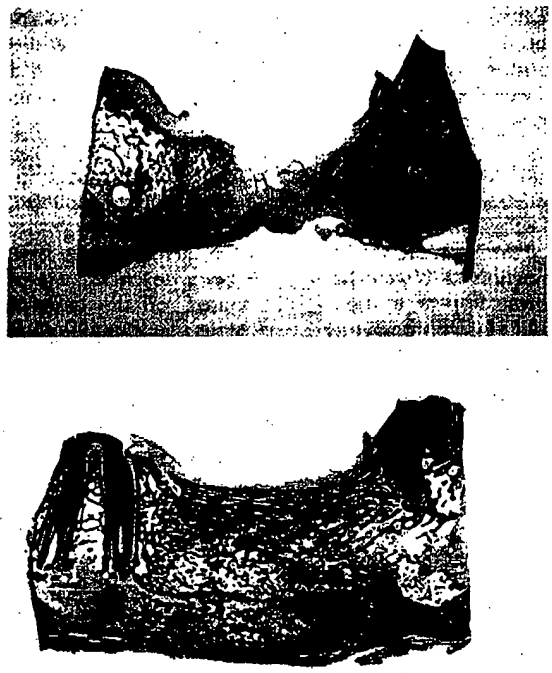

FIG. 1

Photomicrographs of 3 cm mandibular defects in mongrel dogs. Top: 3 months after the 3 cm defect has been made. There is some minimal healing at the edges of the defect but the bone has failed to unite. Bottom: 3 months after the 3 cm defect has been treated with BMP-2, the bone surfaces have reunited and the contour of the bone has returned to normal. Photos courtesy of M. Holtrop.

genes for these proteins has revealed that BMP-2–7 are all synthesized as dimeric precursor glycoproteins that are proteolytically processed before secretion to produce a family of 30 kDa homodimeric proteins[13]. Purified recombinant human BMP-2, -4, -5 and -7 have osteoinductive activity as demonstrated in the ectopic bone formation assay; at present there are only preliminary data in the literature for BMP-3 and none for BMP-6 (Refs 14–16).

Interestingly, BMP-2–7 are members of the transforming growth factor β (TGF-β) gene superfamily, and are most closely related to those molecules thought to be involved in determination of cell fate during development (Fig. 3b). The BMP-2 and -4 genes are 75% identical to the *decapentaplegic* (*dpp*) gene of *Drosophila*. The fundamental role of *dpp* in both dorsoventral body patterning and in imaginal disk formation in *Drosophila* suggests that BMP-2 and -4 may, by analogy, play a role in vertebrate development[17]. The BMP-5, -6 and -7 genes are 70% identical to the recently discovered *Drosophila 60A* gene[18], whose expression pattern suggests that it is involved in multiple stages of *Drosophila* development. BMP-2–7 also share strong homology with Vg1, an mRNA localized to the presumptive endoderm of *Xenopus* oocytes before gastrulation, which has been postulated to be involved in mesoderm specification in the frog[19]. In this context, the BMP-6 gene is the human homologue of the mouse *Vgr-1* gene which was, in turn, discovered by screening a murine cDNA library with a *Xenopus Vg1* probe[20]. These relationships suggest a role for BMP-2–7 in mesoderm specialization among higher animals*.

BMP-2–7 and the activin/inhibins also share considerable homology. In adult mammals, activin/inhibin molecules help regulate erythrocyte differentiation and modulate the release of follicle-stimulating hormone[21]. Interestingly, activin has been identified as the mesoderm-inducing substance purified from *Xenopus* XTC cell-conditioned medium[22,23], providing another example of mesoderm differentiation activity by homologues of the BMPs. Activin mRNAs have recently been localized by *in situ* hybridization to many sites within the developing skeleton[24]. Activin mRNAs appear after BMP-2 and -4 mRNAs, suggesting some later role in skeleton formation. Müllerian inhibitory substance, a more distant relative of the BMPs, is an important morphogenetic signal in developing reproductive systems of mammalian embryos[25].

Finally, the BMPs are 30–40% homologous to members of the TGF-β family, which are present in many embryonic tissues undergoing secondary induction. Most cell types have been shown to possess TGF-β receptors, and the growth factor appears to affect an ever increasing number of regulatory functions in adult organisms[26]. The abundance of TGF-β in bone after osteoblast differentiation suggests it may also be an important regulator of bone remodeling, possibly through its effects on the production of extracellular matrix molecules[27].

Unlike the other BMPs characterized to date, BMP-1 is structurally unrelated to the TGF-β gene superfamily. Recently, it has been shown that the BMP-1 gene is the human homologue of the *Drosophila tolloid* gene[28]. In *Drosophila*, this locus is necessary for proper dorsoventral patterning, and its positional effects are due, in part, to its interaction with DPP, a BMP-2/4 homologue. By analogy, BMP-1 may have a role in embryonic patterning in vertebrates, although its precise role in the developing embryonic skeleton remains to be determined.

In summary, a family of BMPs have been discovered that possess osteoinductive properties in the adult animal and are structurally related to a family of gene products with diverse roles in the developmental programs of many organisms. The evolutionary conservation of these genes among diverse species provides further support for their importance for normal growth and development. While this review focuses on the relevance of BMPs to bone induction, their localizations during embryogenesis suggest a more fundamental role in epithelial–mesenchymal interactions.

*There has been a recent proposal [see K.M. Lyons *et al.* (1991) *Trends Genet.* 7, 408–412] that BMP-2–7 should be renamed DVR-2–7 (for *decapentaplegic-Vg*-related) to emphasize their wider developmental role. To avoid confusion in this review we have retained the designation BMP, since we are specifically discussing the role of these proteins in bone development.

Are the BMPs osteoinductive signals?

Recent evidence from many animal models of bone repair indicates that BMPs are osteoinductive agents in adult animals. In addition to the ectopic induction of bone observed in rats, BMPs have been shown to induce new bone formation in surgically produced bone defects in the rat femur[29], sheep femur[30] and dog mandible[31] (Fig. 4). Moreover, the BMP-induced bone formation appears to follow the sequence of events observed in embryonic endochondral bone formation. Mesenchymal cells are converted into chondroblasts. The cartilage is made, removed, and subsequently replaced with new, functionally mature bone. This bone has the proper size and shape, and undergoes the normal bone remodeling process. These results suggest that BMPs are osteoinductive agents, and their presence in bone provides further evidence that they are the naturally occurring osteoinductive proteins required for bone formation in adults. *In vitro*, BMP-2 appears to induce osteoblast differentiation in two clonal cell lines isolated from mouse bone marrow and calvaria (developing skull), as indicated by increased expression of osteocalcin and alkaline phosphatase, as well as increased sensitivity to parathyroid hormone – all characteristics of the osteoblast phenotype[32,33]. These observations provide further evidence of the osteoinductive properties of BMPs.

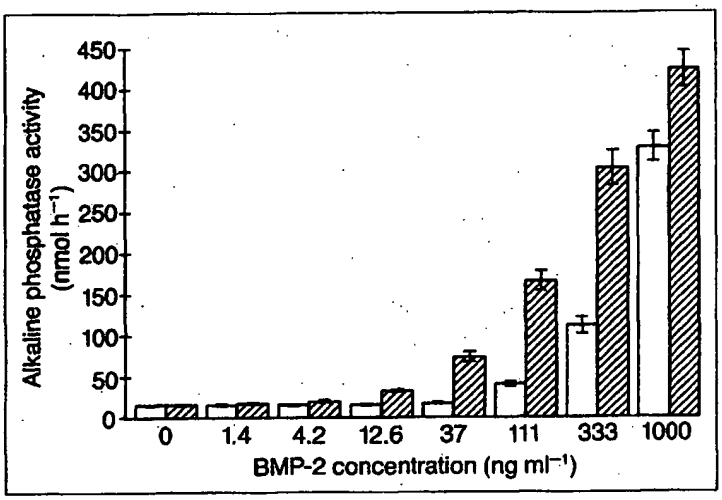

FIG 5

Measurement of alkaline phosphatase activity in a mesenchymal cell line (MLB 13MYC) derived from 13 d.p.c. mouse limb buds. The cells were treated with BMP-2 (open bars) or BMP-2 and 10^{-7} M retinoic acid (hatched bars) for 48 hours and alkaline phosphatase activity was measured spectrophotometrically by the amount of *p*-nitrophenol produced from *p*-nitrophenol phosphate. The data presented are the mean plus or minus standard error of triplicate samples; note log scale on horizontal axis.

There is also evidence to suggest that the BMPs are the osteoinductive signals involved in embryonic skeleton formation. BMP-2, -4 and -6 have been localized by *in situ* hybridization to areas undergoing skeleton formation in the embryonic mouse[34,35]. The localizations seen are consistent with the involvement of BMP-2 and -4 in limb pattern formation and limb osteogenesis. The temporal expression of BMP-2 and -4 further suggests that they act at the same time as retinoic acid (RA) and possibly other retinoids, during the period of Hox gene expression. BMP-6 is expressed in the developing skeleton at later times, and may be an important modulator of the final events in skeleton development. Analysis of the expression patterns of BMP-3, -5 and -7 is underway and may provide more information on the local signals present throughout skeleton development. It is possible that the observed differences in the location and temporal expression of the BMP mRNAs indicate that, as a group, these molecules may be cues for the sequential morphogenetic processes involved in creating the skeleton.

While there is no direct evidence to show that the BMPs are necessary for embryonic bone formation, data obtained from cultured embryonic bone cells *in vitro* demonstrate that BMPs affect the growth and differentiation of these cells. For example, in multipotential embryonic calvarial cells BMP-2 stimulates production of alkaline phosphatase, an enzyme associated with the osteoblast phenotype, and depresses expression of desmin, a gene product associated with muscle phenotype[32]. BMP-2 treatment can also increase alkaline phosphatase activity and sensitivity to parathyroid hormone in limb bud cells[36,37], suggesting that BMP-2 influences the development of osteoprogenitor cells in the limb (Fig. 5).

Although the role of BMPs in embryonic development is unknown, it is interesting to speculate that they may be involved in regulation of Hox gene expression[38], perhaps by modulating the regulatory effects of RA and its receptors. In support of this hypothesis is the observation that BMP-2 induces expression of the β form of the RA receptor in F9 cells[39]. There is also preliminary evidence for interactive effects between BMP-2 and RA in mouse limb bud cell lines, where RA modulates BMP-2-induced cell differentiation (V. Rosen, unpublished).

Summary and conclusions

The BMPs are osteoinductive agents present in adult bone. When these proteins are added exogenously to both bony and nonbony sites they are potent initiators of new bone formation through a process that appears very similar to the endochondral bone formation seen during embryonic skeletal development. The BMPs appear to act on mesenchymal progenitor cells, directing their differentiation into both cartilage and bone-forming cells.

BMPs are present in the embryo at sites of skeleton formation, and have been shown to regulate both the growth and differentiation of embryonic skeletal precursor cells *in vitro*. This, coupled with the homology of the BMPs to regulatory molecules important for embryonic development, suggests that the BMPs function as information molecules during embryonic skeleton formation. The precise manner in which regulation of

mesenchymal stem cells by BMPs might occur is speculative. One possibility is that BMPs interact with positional cues in the embryo to correlate cell differentiation and pattern formation. In support of this idea, BMPs and RA are known to have cooperative or interdependent effects *in vitro*. This effect might be direct or may involve multiple sets of regulatory molecules. The similarities between components of new bone formation at fracture sites in the adult skeleton and initial bone formation in the embryonic skeleton also suggest a common mechanism regulated by the same sets of control molecules. Additional studies of the effects and mechanisms of action of BMPs may increase our understanding of bone formation and repair, and the complex events underlying these processes.

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V. Rosen and R.S. Thies are in the Genetics Institute Inc., 87 Cambridge Park Drive, Cambridge, MA 02140-2387, USA.

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Cartilage-derived Morphogenetic Proteins

NEW MEMBERS OF THE TRANSFORMING GROWTH FACTOR-β SUPERFAMILY PREDOMINANTLY EXPRESSED IN LONG BONES DURING HUMAN EMBRYONIC DEVELOPMENT*

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Steven C. Chang‡, Bang Hoang§, J. Terrig Thomas, Slobodan Vukicevic, Frank P. Luyten¶, Nicholas J. P. Ryba‖, Christine A. Kozak, A. Hari Reddi‡‡, and Malcolm Moos, Jr.§§**

*From the Bone Research Branch and the ‖Laboratory of Immunology, NIDR, National Institutes of Health, Bethesda, Maryland 20892, the §§Laboratory of Developmental Biology, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892, the ‡‡Laboratory of Musculoskeletal Biology, Department of Orthopedic Surgery, Johns Hopkins School of Medicine, Baltimore, Maryland 21205, and the **Laboratory of Molecular Microbiology, NIAID, National Institutes of Health, Bethesda, Maryland 20892*

Partially purified extracts from newborn calf articular cartilage were found to induce cartilage and bone when subcutaneously implanted in rats. This activity showed characteristics of bone morphogenetic proteins (BMPs). Degenerate oligonucleotide primer sets derived from the highly conserved carboxyl-terminal region of the BMP family were designed and used in reverse transcription-polymerase chain reactions with poly(A)⁺ RNA from articular cartilage as template to determine which BMPs are produced by chondrocytes. Two novel members of the transforming growth factor-β (TGF-β) superfamily were identified and designated cartilage-derived morphogenetic protein-1 (CDMP-1) and -2 (CDMP-2). Their carboxyl-terminal TGF-β domains are 82% identical, thus defining a novel subfamily most closely related to BMP-5, BMP-6, and osteogenic protein-1. Northern analyses showed that both genes are predominantly expressed in cartilaginous tissues. *In situ* hybridization and immunostaining of sections from human embryos showed that CDMP-1 was predominantly found at the stage of precartilaginous mesenchymal condensation and throughout the cartilaginous cores of the developing long bones, whereas CDMP-2 expression was restricted to the hypertrophic chondrocytes of ossifying long bone centers. Neither gene was detectable in the axial skeleton during human embryonic development. The cartilage-specific localization pattern of these novel TGF-β superfamily members, which contrasts with the more ubiquitous presence of other BMP family members, suggests a potential role for these proteins in chondrocyte differentiation and growth of long bones.

The discovery of a family of bone morphogenetic proteins (BMPs),[1] which can induce endochondral bone formation in adult animals (1–6), suggested that BMP inductive signals may be intimately involved in both the development and regeneration of the skeleton. The BMPs belong to the TGF-β superfamily, whose members are widely represented throughout the animal kingdom. This superfamily comprises a large group of structurally related signaling proteins that are secreted as dimers and then cleaved after an Arg-*X*-*X*-Arg site to release biologically active carboxyl-terminal domains containing seven highly conserved cysteines. These proteins have different roles at various stages of embryogenesis and in adult animals (7–9). Several BMPs produced as recombinant proteins induce endochondral bone formation when subcutaneously implanted in rats (2, 10, 11). This apparent redundancy of activity suggests that the actual physiological roles for the various members of the family remain to be determined.

BMPs may have wide-ranging extraskeletal roles in development, as suggested by the following observations. 1) Localization studies in both human and mouse tissues have demonstrated high levels of mRNA expression and protein synthesis for various BMPs in kidney (BMP-3, -4, and -7), lung (BMP-3, -4, -5, and -6), small intestine (BMP-3 and -7), heart (BMP-2, -4, -6, and -7), limb bud (BMP-2, -4, -5, and -7), and teeth (BMP-3, -4, and -7) (12–17). 2) Several members of the family, including BMP-4 and -7, are key molecules in epithelia-mesenchyma interactions, for example during odontogenesis (18, 19). 3) BMP-2 and -4 are involved in the signaling pathway that controls patterning in the developing chick limb (20), and BMP-4 is a ventralizing factor in early *Xenopus* development (21, 22). 4) The homologues of the BMPs in *Drosophila*, the decapentaplegic (*dpp*) and 60A (23, 24) gene products, have the capacity to induce bone in mammals (25), whereas human BMP-4 is able to confer the normal embryonic dorso-ventral patterning in *Drosophila* transformants defective in expressing *dpp* (26).

The defects in skeletal structures caused by mutations and deletions of the *Bmp5* gene in short ear mice (27) support the hypothesis that at least some members of the family are required for proper patterning in the vertebrate limb and in other skeletal structures. It is surprising that known BMPs, which by definition are intimately involved in cartilage and bone formation in experimental model systems, are not expressed at significant levels in the chondroblasts and chondrocytes of the cartilage core of developing long bones except in the hypertrophic chondrocytes, where both Vgr-1 (BMP-6) (13) and OP-1 (BMP-7) (16, 17) have been found. In this study, we show that

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U13660 and U13661.

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¶ To whom correspondence should be addressed: Developmental Biology Program, Bone Research Branch, NIDR, National Institutes of Health, Bldg. 10, Rm. 1N108, Bethesda, MD 20892. Tel.: 301-402-3502; Fax: 301-480-2012.

[1] The abbreviations used are: BMPs, bone morphogenetic proteins; CDMP, cartilage-derived morphogenetic protein; RT-PCR, reverse transcription-polymerase chain reactions; TGF-β, transforming growth factor-β; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid; kb, kilobase(s); bp, base pair(s).

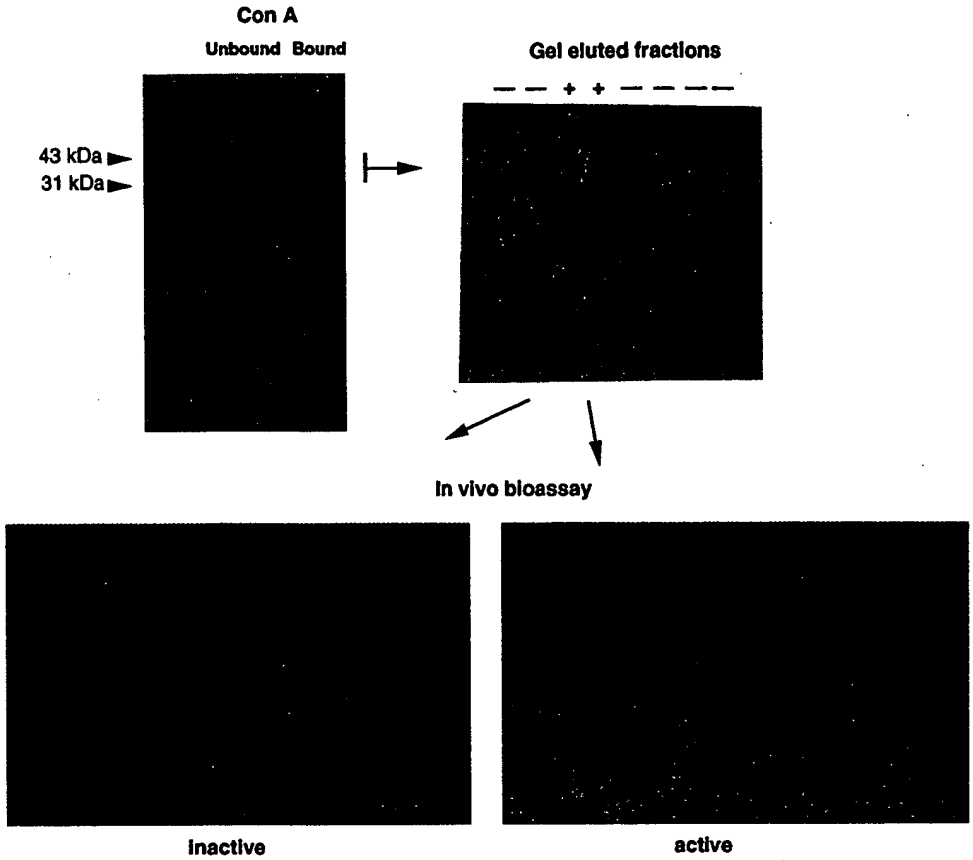

FIG. 1. **Partial characterization of articular cartilage-derived chondrogenic activity.** 1.2 M guanidine HCl extracts from articular cartilage were partially purified by heparin-Sepharose affinity chromatography, molecular sieve chromatography (S-200 HR), and concanavalin A (*Con A*) affinity chromatography as described under "Materials and Methods." Aliquots of concanavalin A-bound and unbound material were electrophoresed on 12% SDS-polyacrylamide gels and stained with silver. Preparative SDS-gel electrophoresis and electro-elution of the concanavalin A-bound material was performed, and one-third of the eluates were bioassayed by ethanol precipitation on insoluble collagenous residue of rat-demineralized bone matrix as a carrier. The resulting pellets were subcutaneously implanted in Long Evans rats and harvested after 10 days. The specimens were then fixed in Bouin's reagent, embedded in plastic, and stained with toluidine blue (*bottom*). Metachromatic staining of cartilage matrix indicates biological activity in the gel-eluted fractions (+).

extracts of articular cartilage have bone-inductive activity and describe two new putative chondrogenic members of the TGF-β superfamily, designated cartilage-derived morphogenetic protein-1 (CDMP-1) and -2 (CDMP-2), which are preferentially expressed in cartilaginous tissues.

MATERIALS AND METHODS

Partial Characterization of the Chondrogenic Activity in Cartilage—Articular (metatarsophalangeal joints), scapular, and nasal cartilage (300 g, wet weight, per tissue) were prepared from newborn calves. Epiphyseal cartilage was dissected from fetal bovine femurs (7–8 months). The tissues were finely minced and homogenized with a Polytron (top speed, 2 × 30 s) in 20 volumes of 1.2 M guanidine HCl, 0.5% CHAPS, 50 mM Tris-HCl, pH 7.2, containing protease inhibitors and extracted overnight at 4 °C as previously described (3). These conditions extract >90% of the lower molecular weight matrix while leaving most of the high molecular weight proteoglycans behind (28). The extracts were concentrated and exchanged with 6 M urea by diafiltration using an Ultrasette™ (Filtron Technology Inc.) and loaded on a 0.5-liter heparin-Sepharose (Pharmacia Biotech Inc.) column. Thereafter, the column was washed with 5 bed volumes of 6 M urea, Tris-HCl, pH 7.4, with 0.15 M NaCl and then eluted with 2 volumes of 1 M NaCl in the same buffer (3). Chondrogenic activity was assayed by reconstituting a portion of the eluate with 25 mg of guanidine-insoluble collagenous residue of demineralized rat bone matrix (3). The implants were recovered after 10 days, and alkaline phosphatase activity was measured as previously described (3). The specific activity was expressed as units of alkaline phosphatase/mg of protein used for reconstitution in the bioassay. Implants were also examined by histology. The 1 M NaCl eluate of articular cartilage, which contained biological activity, was concentrated by diafiltration and loaded onto a Sephacryl S-200 HR gel filtration column (XK 50/100, Pharmacia Biotech Inc.) as previously reported (3). After molecular sieve chromatography, the bioactive fractions were pooled and exchanged with 50 mM Hepes, pH 7.4, containing 0.15 M NaCl, 10 mM $MgSO_4$, 1 mM $CaCl_2$, and 0.1% (w/v) CHAPS using Macrosep™ concentrators (Filtron Technology Inc., Northborough, MA). The equilibrated sample was mixed with 1 ml of concanavalin A-Sepharose (Pharmacia Biotech Inc.) previously washed with 20 volumes of the same buffer (29). After overnight incubation on an orbital shaker at 4 °C, the slurry was packed into a disposable 0.7-cm inner diameter Bio-Rad column and washed with 20 volumes of the Hepes buffer to remove unbound proteins. Bound proteins were then eluted with 20 volumes of the same buffer containing 500 mM methyl-D-mannopyranoside. The eluate was concentrated to 200 μl using Macrosep™ concentrators. Macromolecules were then precipitated overnight with 9 volumes of absolute ethanol at 4 °C. The precipitate was redissolved in 1 ml of 6 M urea, Tris-HCl, pH 7.4, and bioassayed for cartilage and bone inducing activity by subcutaneous implantation with collagen carrier in rats both before and after reduction and alkylation with dithiothreitol and iodoacetamide (3). The bioactive bound protein was then mixed with 2 × Laemmli sample buffer (30) without reducing agents and electrophoresed on a 12% preparative SDS-polyacrylamide gel. Gel elution of the separated protein fractions was performed as previously described (3), and the gel-eluted fractions were tested for biological activity.

RT-PCR Using Degenerate Oligonucleotide Primers—Total RNA from bovine articular chondrocytes was extracted using a modified acid guanidine-phenol-chloroform method (31, 32). Poly(A)$^+$ RNA was isolated using magnetic beads (Poly(A)Tract™, Promega, Madison, WI). Four degenerate oligonucleotide primers corresponding to highly conserved motifs in the carboxyl-terminal region of the BMPs were used: S1, 5′-GGI-TGG-(C/A)AI-GA(C/T)-TGG-AT(A/C/T)-(A/G)TI-GC(A/C/G/T)CC-CC-3′ corresponding to amino acids GW(Q/N)DWI(I/V)AP; S2, 5′-GGI-TGG-(A/T)(G/C)(I)-GA(GA)-TGG-AT(T/C/A)-ATI-(A/T)G(A/C/G/T)-CC-3′ corresponding to amino acids GWSEWIISP; AS1, 5′-A(A/G)-(A/G)GT-(C/T)TG-(A/C/G/T)AC-(A/G)AT-(A/G)GC-(A/G)TG-(A/G)TT-3′ correspond-

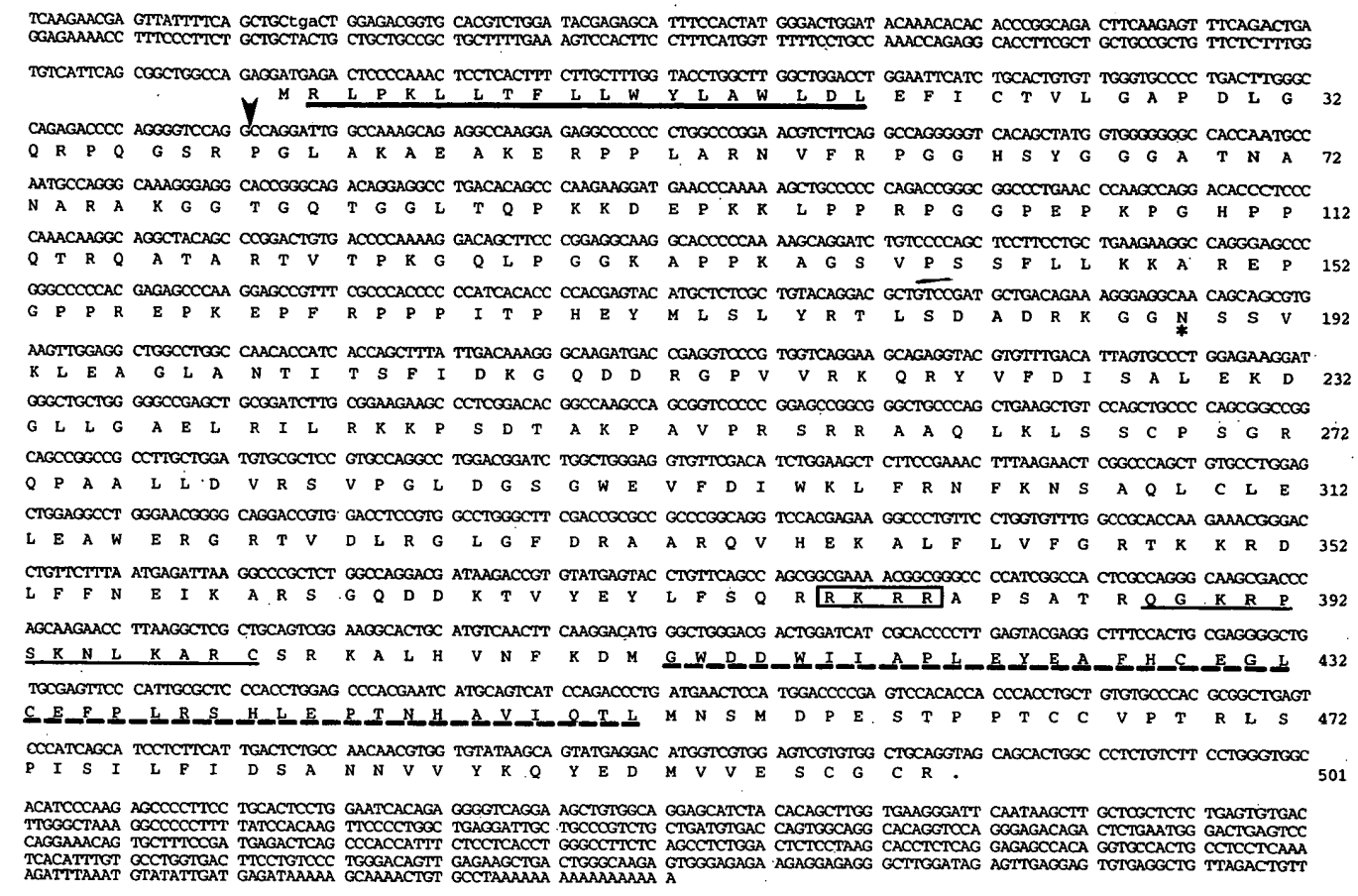

FIG. 2. **Nucleotide and amino acid sequence of the full-length human CDMP-1 cDNA.** The predicted CDMP-1 product contains 500 amino acids with a putative proteolytic processing site (RXXR/A, *box*) preceding a 120-amino acid mature carboxyl-terminal region. A single *N*-linked glycosylation site is located in the pro-region (*asterisk*). The putative signal peptide (42) is *underlined* in *bold*. A termination codon (TGA) is shown in the 5′-untranslated region. The *bold dashed underline* indicates the fragment obtained by RT-PCR and used to screen cDNA libraries. The 13-amino acid peptide used to raise a polyclonal antibody in rabbits is *underlined*. A *vertical arrowhead* marks the boundary between sequence obtained from genomic DNA and cDNA.

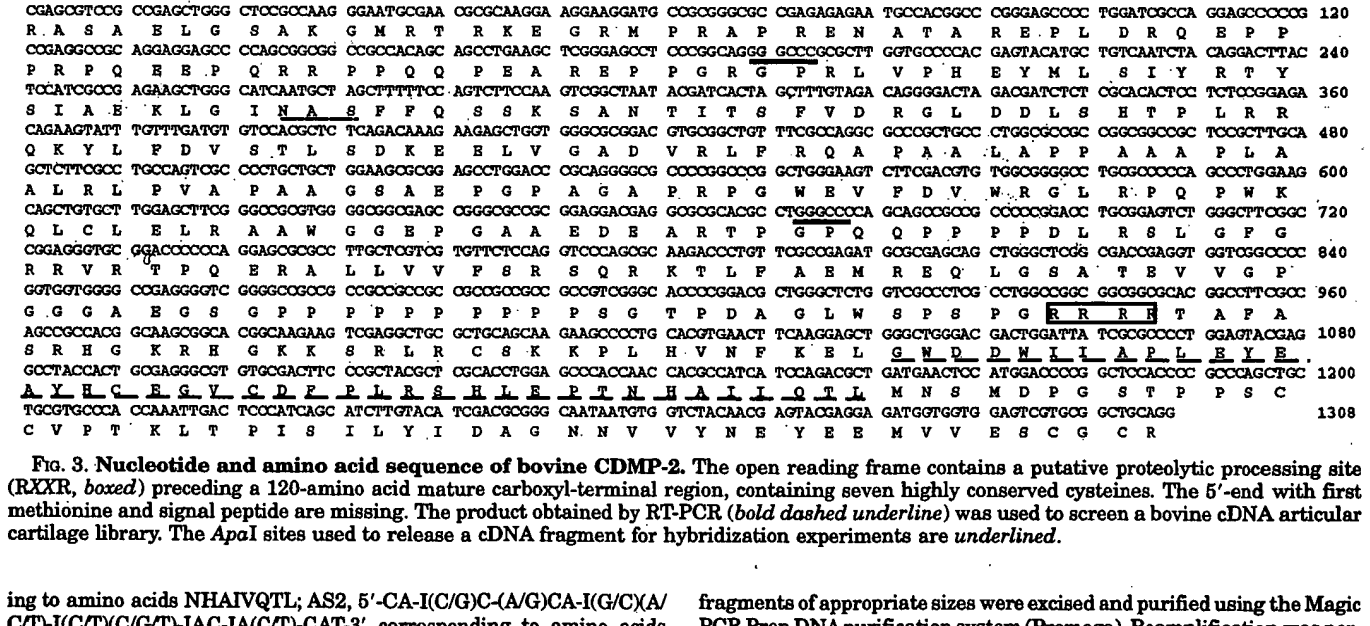

FIG. 3. **Nucleotide and amino acid sequence of bovine CDMP-2.** The open reading frame contains a putative proteolytic processing site (RXXR, *boxed*) preceding a 120-amino acid mature carboxyl-terminal region, containing seven highly conserved cysteines. The 5′-end with first methionine and signal peptide are missing. The product obtained by RT-PCR (*bold dashed underline*) was used to screen a bovine cDNA articular cartilage library. The *Apa*I sites used to release a cDNA fragment for hybridization experiments are *underlined*.

ing to amino acids NHAIVQTL; AS2, 5′-CA-I(C/G)C-(A/G)CA-I(G/C)(A/C/T)-I(C/T)(C/G/T)-IAC-IA(C/T)-CAT-3′ corresponding to amino acids M(V/I)V(E/R)(G/S/A)C(G/A)C. Nucleotides in parenthesis denote sites of degeneracy, and I denotes inosine. First strand cDNA synthesis was performed using 1 μg of Poly(A)$^+$ or 5 μg total RNA with oligo(dT), random hexanucleotide primers, or the antisense degenerate primers, AS1 and AS2. The PCR amplifications with the degenerate sense primers, S1 and S2, were performed using conditions previously described (24). The reaction products were electrophoresed on 1.2% agarose gels, and DNA fragments of appropriate sizes were excised and purified using the Magic PCR Prep DNA purification system (Promega). Reamplification was performed with the same primers, and each PCR product was subcloned into the PCR II vector using the TA Cloning™ system (Invitrogen).

Library Screening—A 120-bp PCR fragment encoding part of the carboxyl-terminal domain of novel BMP-like genes (Fig. 2, *dashed line*) was used to screen two cDNA libraries. One library, from adolescent human articular cartilage poly(A)$^+$ RNA (kindly provided by Dr. Björn Olsen, Harvard, Boston, MA), was primed with oligo(dT) and con-

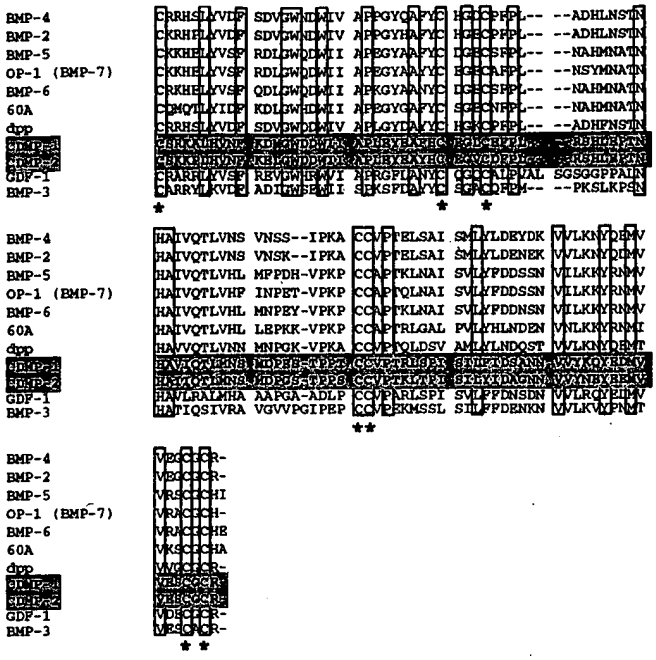

FIG. 4. **Sequence alignments of the carboxyl-terminal domains.** The amino acid sequence of CDMP-1 and CDMP-2 (*bold, shaded*) are aligned with the most closely related TGF-β superfamily members. The completely conserved residues are boxed. *Dashes* indicate gaps introduced for alignment.

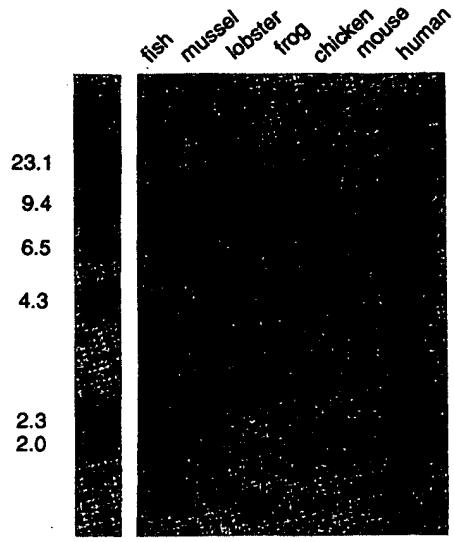

FIG. 5. **Southern blot analysis of evolutionary relatedness.** Southern hybridization was performed using an *Apa*I cDNA fragment (0.685 kb) of the pro-region of CDMP-1. A panel of *Eco*RI-restricted genomic DNAs was used; from *left* to *right*, fish (*T. onitis*), mussel (*M. edulis*), lobster (*H. americanus*), frog (*X. laevis*), chicken (*G. domesticus*), mouse (*M. musculus*), and human (*H. sapiens*). Size markers (in kb) are indicated on the *left*.

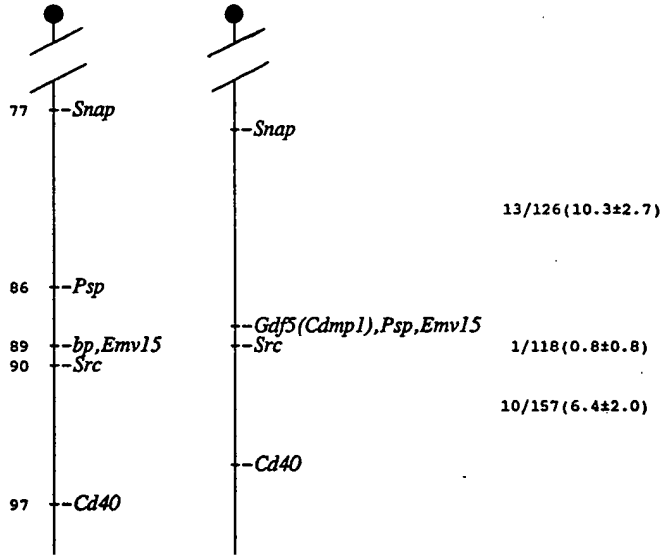

FIG. 6. **Genetic maps of chromosome 2 showing the location of *Cdmp1*.** The map on the *right* is based on the data from two separate crosses. Recombination fractions are given to the *right* of each map for each adjacent locus pair or cluster. *Numbers* in *parentheses* represent the percent recombination and standard error calculated according to Green (43). The map on the left is an abbreviated version of the chromosome 2 committee map (48) and shows the map location of brachypodism (*bp*) relative to the other markers typed in the crosses used here.

structed in the λgt11 vector. The other was a bovine oligo(dT) and random-primed articular cartilage cDNA library constructed in the UNIZAP™XR vector (Stratagene). Approximately 1×10^6 plaques from each library were screened by standard procedures (33). Hybridizations were performed for 20 h at 42 °C in 6 × SSC, 1 × Denhardt's solution, 0.01% tRNA, 0.05% sodium pyrophosphate, and the membranes (137-mm nylon membranes, DuPont NEN) were washed to final stringency of 6 × SSC, 0.1% SDS at 55 °C for 20 min.

The majority of the open reading frame and the 3′-untranslated region of CDMP-1 was derived from cDNA clones originally isolated from the human articular cartilage library. The 5′-end, which was lacking in the original cDNA clones, was subsequently obtained from a human genomic library in the EMBL-3 vector (Clontech). The same screening conditions were also used to isolate a 2.8-kb CDMP-2 cDNA clone from the bovine articular cartilage library.

DNA Sequencing—All sequencing was done by dideoxy DNA sequencing (34) of both strands using Sequenase version 2.0 DNA polymerase according to the manufacturer's instructions (U. S. Biochemicals) with at least 2-fold redundancy. Confirmatory data in ambiguous regions were obtained by automated thermal cycle sequencing with an Applied Biosystems model 370A sequencer and by using 7-deaza-GTP (U. S. Biochemicals). The sequencing data were obtained from restriction fragments subcloned into pBluescript using either M13 forward and reverse primers or synthetic oligonucleotide primers.

Northern and Southern Hybridization—For Northern blot analyses, equal amounts of poly(A)$^+$ RNA (2 μg) were electrophoresed on 1.2% agarose-formaldehyde gels and transferred to Nytran membranes (Schleicher & Schuell) (33). Multiple tissue Northern blots were obtained from Clontech. The membranes were prehybridized for 3 h at 42 °C in hybridization buffer (5 × SSPE, 5 × Denhardt's solution, 50% formamide, 1% SDS, and 100 μg/ml freshly denatured salmon sperm DNA). Hybridizations with [^{32}P]dCTP-labeled probes, having specific activities of at least 1×10^9 cpm/μg, were performed overnight under the same conditions as the prehybridization. Probes included the cDNA probe for human glyceraldehyde-3-phosphate dehydrogenase (1.1 kb) (Clontech), an *Apa*I fragment (bp 470–1155) of CDMP-1, and an *Apa*I fragment (bp 194–677) of CDMP-2. The CDMP-1 and CDMP-2 probes were chosen to avoid the highly conserved carboxyl-terminal domain, thereby minimizing the potential for cross hybridization with other members of the gene family. Following hybridization, the filters were washed to a final stringency of 55 °C, 0.4 × SSC, 0.1% SDS. The expression levels were quantified using a Phosphorimager (Molecular Dynamics).

Southern hybridization was performed using the evolutionary relatedness blot (Bios Laboratories, New Haven, CT) under the conditions recommended by the manufacturer. The panel of *Eco*RI-digested genomic DNAs included human (*Homo sapiens*), mouse (*Mus musculus*), chicken (*Gallus domesticus*), frog (*Xenopus laevis*), lobster (*Homarus americanus*), mussel (*Mytilus edulis*), fish (*Tautoga onitis*), fruit fly (*Drosophila melanogaster*), nematode (*Caenorhabditis elegans*), yeast (*Saccharomyces cerevisiae*), and bacteria (*Escherichia coli*). The 2.1-kb CDMP-1 *Eco*RI fragment originally obtained from the cDNA library was used as a probe, and the blot was washed to a final stringency of 0.4 × SSC, 0.1% SDS at 55 °C.

Genetic Mapping—The *Apa*I fragment (see above) of CDMP-1 was used as a hybridization probe on Southern blots to type DNAs from two genetic crosses: (NFS/N or C58/J × *M. musculus musculus*) × *M. musculus musculus* (35) and (NFS/N × *Mus spretus*) × *M. spretus* or C58/J (36). DNAs from these crosses have been typed for over 650 markers

including the chromosome 2 markers *Snap* (synaptosomal associated protein 25), *Psp* (parotid secretory protein), *Emv15* (ecotropic murine leukemia virus 15), *Src* (*src* oncogene), and *Cd40* (cluster designation 40). Probes for these markers and restriction fragment length polymorphisms used to type these crosses have been previously described (35, 37). *Src* was typed using a mouse Src probe obtained from E. Rassart (University of Quebec, Montreal) following *Xba*I digestion in the *musculus* cross and *Bam*HI digestion in the *spretus* cross.

In Situ Hybridization—Tissues from human embryos obtained after pregnancy termination ranged from 5 to 14 weeks of gestation, estimated in weeks on the basis of crown-rump length and pregnancy records of the conceptual age. They were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2), embedded in paraffin, sectioned serially at 5–7 μm, and mounted on silanated slides. The tissues used in the present study were obtained from legally sanctioned procedures performed at the University of Zagreb Medical School. The procedure for obtaining the human autopsy material was approved and controlled by the Internal Review Board of the Ethical Committee at the School of Medicine, University of Zagreb, and Office of Human Subjects Research at the National Institutes of Health (Bethesda, MD). *In situ* hybridization was done as previously described (15, 38). Briefly, sections were incubated overnight at 50 °C in a humidified chamber in 50% formamide, 10% dextran sulfate, 4 × SSC, 10 mM dithiothreitol, 1 × Denhardt's solution, 500 μg/ml freshly denatured salmon sperm DNA, and yeast tRNA with 0.2–0.4 ng/ml ^{35}S-labeled riboprobe (1 × 10^9 cpm/μg). *Apa*I fragments of CDMP-1 and CDMP-2 (described above) from the pro-region, subcloned in both sense and antisense direction into pBluescript II SK(+) vector (Stratagene), were used as transcription templates. Riboprobes were then prepared using T7 RNA polymerase (Sure Site kit, Novagen, Madison, WI) according to the manufacturer's instructions and used with and without prior alkaline hydrolysis. After hybridization, the sections were washed as described (13) to a final stringency of 0.1 × SSC, 65 °C for 2 × 15 min. After dehydration through a graded ethanol series containing 0.3 M ammonium acetate, slides were covered with NTB-2 emulsion (Kodak) and exposed between 1 and 3 weeks. After development, the slides were stained with 0.1% toluidine blue, dehydrated, cleared with xylene, and mounted with Permount.

Immunostaining—A polyclonal antibody to the peptide QGKRPSKNLKARC (amino acids 388–400, prepared by Peptide Technologies, Gaithersburg, MD), which belongs to the mature secreted protein of CDMP-1, was raised in rabbits. Before immunization, the peptide was conjugated to Imject® maleimide-activated keyhole limpet hemocyanin (Pierce). Searches performed using the BLAST (39) network service (available through the National Center for Biotechnology Information) indicated that the peptide does not show sequence identity with any known protein or BMP. The embryonic tissue sections were stained as recommended by the manufacturer using immunogold as a detection system (Auroprobe LM, Janssen, Belgium) and counterstained with 0.1% toluidine blue. The primary antibody (crude antiserum) was used at a concentration of 15 μg/ml in phosphate-buffered saline with 0.5% bovine serum albumin for 1 h. In the controls, the primary antibody was replaced with bovine serum albumin, normal rabbit serum, or secondary antibody alone.

RESULTS

Partial Characterization of Cartilage-derived Chondrogenic Activity—Each of the crude extracts of the different cartilaginous tissues (articular, nasal, scapular, or epiphyseal) were inactive when tested directly in the *in vivo* cartilage and bone-inducing assay. However, after heparin affinity chromatography (40), chondrogenic activity was recovered in the 1 M NaCl eluate from both articular and epiphyseal cartilage extracts (data not shown). No activity was detected in the extracts of the other cartilaginous tissues. The specific activity for articular cartilage was the highest (1 unit of alkaline phosphatase/mg protein), and this material was used for characterization of the activity. Further purification of the active fraction by molecular sieve chromatography on Sephacryl S-200 HR (specific activity, 112 units/mg) and affinity chromatography on concanavalin A (specific activity, 480 units/mg) established the presence of cartilage and bone-inducing activity characteristic of the members of the BMP family (Fig. 1). Gel elution experiments with the concanavalin A-bound bioactive fraction demonstrated that the activity resided between 34 and 38 kDa (Fig. 1; the specific activity of the gel-eluted fractions was 2143 units/mg). In addition, the loss of activity following reduction and alkylation (data not shown) suggested that the bioactivity was induced by either a known or a new member(s) of the BMP family.

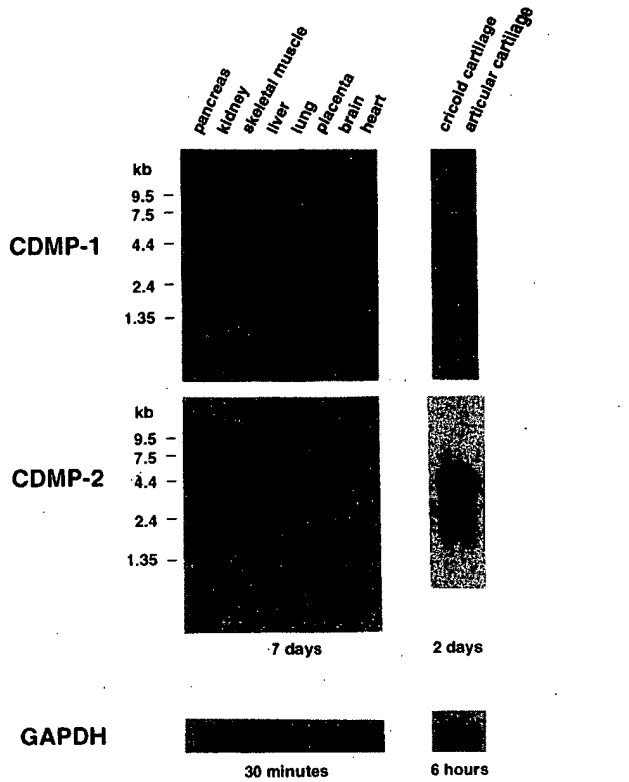

FIG. 7. **Multiple tissue Northern blot analysis of CDMP-1 and CDMP-2.** Equal amounts of poly(A$^+$) RNA (2 μg) were loaded into each lane of 1.2% agarose-formaldehyde gels. Hybridization was performed with specific *Apa*I fragments of the pro-regions. Size markers are indicated on the *left*. Hybridization with the reference probe glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) to check for consistent loading is shown at the *bottom*. Exposure times to Kodak XAR-5 films are indicated in minutes, hours, or days.

Cloning of New Cartilage-derived Morphogenetic Proteins—Following RT-PCR with poly(A)$^+$ RNA from newborn bovine articular cartilage as template and sets of degenerate oligonucleotide primers (S1/AS1 and S1/AS2), amplification products of 120 and 280 bp were obtained (data not shown). Subcloning and sequencing showed that these products encoded BMP-2, BMP-6, BMP-7 (OP-1), and several other BMP-like sequences. Cloned inserts with novel BMP-like sequences were isolated, radiolabeled, and used to screen both a human and a bovine articular cartilage cDNA library. Six clones were isolated from the human cDNA library, and the sizes of the *Eco*RI inserts (2.1 kb) and their restriction maps were found to be identical. One of the clones was sequenced. An open reading frame encoding a BMP-related protein, designated CDMP-1, was identified. It appeared that the human cDNA clone lacked the coding region for the first methionine and signal peptide. This 5′-end of the human CDMP-1 was subsequently obtained from a human genomic clone as described under "Materials and Methods" and contained a consensus translation initiation sequence (41) immediately followed by a putative transmembrane signal sequence (42). The entire open reading frame of CDMP-1 (Fig. 2) contains 500 amino acids consisting of a pro-region of 376 amino acids, a typical cleavage site (R*X*X*R*/A), and a carboxyl-terminal domain of 120 amino acids containing the seven highly conserved cysteines characteristic of the TGF-β gene family.

Two clones with inserts of 2.8 kb were isolated from a bovine

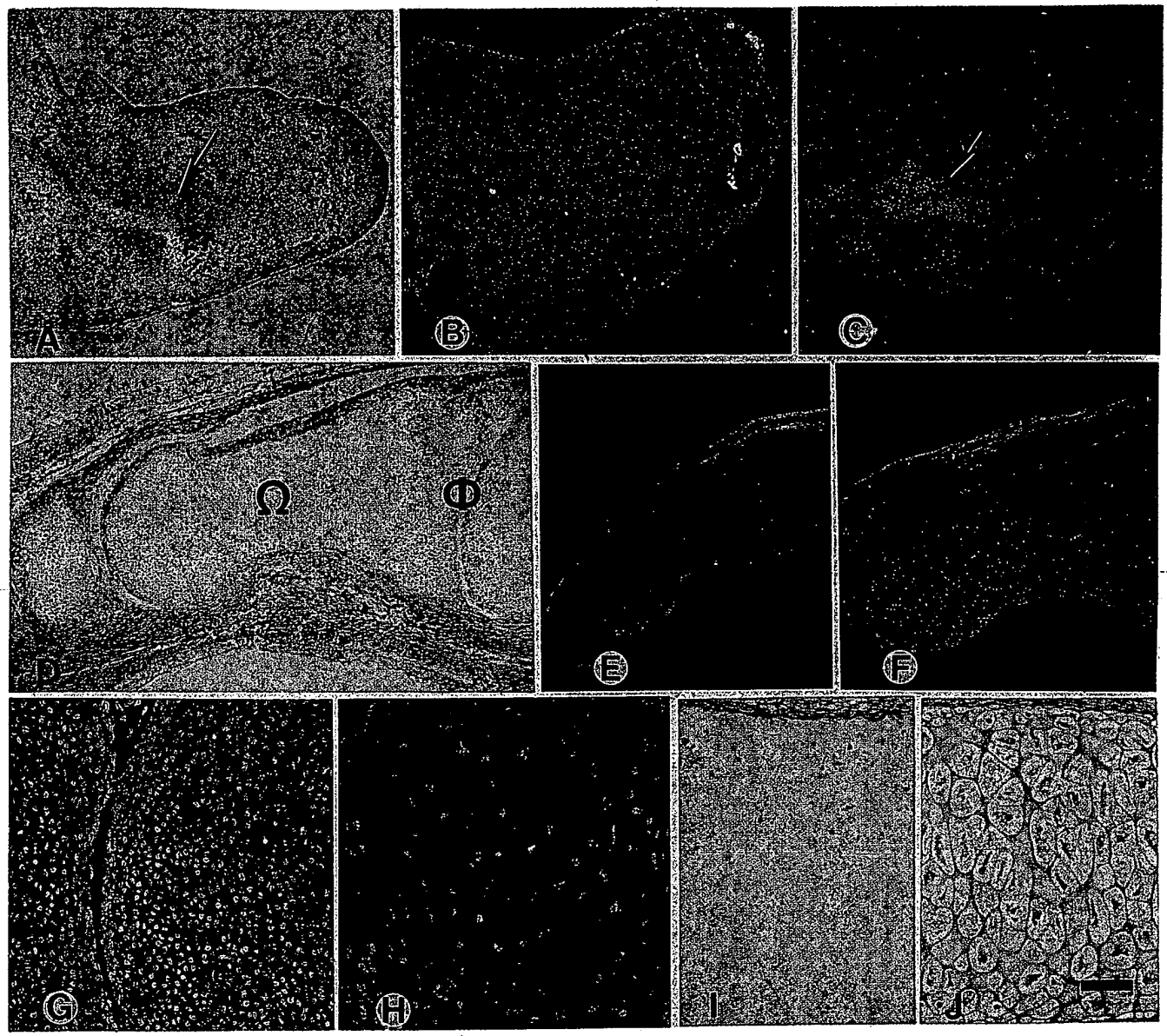

FIG. 8. **CDMP-1 is preferentially localized in postnatal cartilage and the cartilaginous cores of long bones in human development.** Bright (*A*) and corresponding dark field images of 6-week-old human embryonic hind limb sections hybridized with the CDMP-1 sense (*B*) and antisense (*C*) riboprobe. Expression is localized in the precartilaginous mesenchymal condensation, indicated by *arrows*. *D*, bright field image of a section from a digit of a 10-week-old embryo hybridized with the same sense (*E*) and antisense (*F*) riboprobe. *G* and *H*, higher magnifications of corresponding dark fields as indicated by Φ and Ω, respectively, in *D*. Corresponding bright field images of 9-week long bone sections incubated with pre-immune (*I*) and CDMP-1 (*J*) antipeptide polyclonal antiserum. Sections *A*, *E–G*, and *H* were stained with 0.1% toluidine blue, *B* and *C* are unstained, and *D*, *I*, and *J* were stained with 0.2% light green. *Bar* indicates 120 μm in *A–D* and *F*, 200 μm in *E*, 50 μm in *G*, and 25 μm in *H–J*.

articular cartilage cDNA library. Both clones were sequenced, and the open reading frame was found to encode another novel TGF-β-related protein, designated CDMP-2 (Fig. 3). At the 5′-end, the pro-region lacked the first methionine and signal peptide. The mature carboxyl-terminal domain of 120 amino acids showed 82% identity with CDMP-1 (Fig. 4). Alignment of the carboxyl-terminal domains of CDMP-1 and CDMP-2 with other members of the BMP family revealed an amino acid identity of about 50% with BMP-5, BMP-6, and OP-1 (BMP-7) (Fig. 4). These results suggest that CDMP-1 and CDMP-2 are members of a new subfamily.

Genetic Mapping of CDMP-1—Southern analyses in evolutionary relatedness blots, using the original 2.1-kb human CDMP-1 cDNA probe (starting from amino acid 40, see Fig. 2), showed 5.9- and 2.6-kb bands in humans (Fig. 5) and strong hybridization in both mouse and chicken. Fainter bands were seen in fish, frog, and lobster after 5 days of autoradiographic exposure. Sequences amplified from *X. laevis* and *Danio rerio* in the conserved carboxyl-terminal domain showed 95% identity to this gene, confirming the high degree of conservation throughout the vertebrates.[2] No hybridization was detected to *Drosophila* DNA (data not shown). Southern blot hybridization with the same 2.1-kb cDNA as described above was used to map the gene in the mouse. This probe identified *Eco*RI fragments of 7.1 and 2.0 kb in *M. spretus* and *M. musculus musculus* and 6.8 and 3.2 kb in NFS/N and C58/J.

Inheritance of the polymorphic fragments in the progeny of the two crosses used for mapping was compared with inheritance of over 650 markers previously mapped to all 19 autosomes and the *X* chromosome. As shown in Fig. 6, the gene

[2] L. Kleinwaks, M. Krinks, and M. Moos, unpublished observations.

encoding CDMP-1 was linked to markers on chromosome 2 just proximal to *Src*. The closest linkage was observed with *Psp* and *Emv15*. No recombination was observed between *Cdmp1* and *Psp* in the 100 mice typed for both markers, indicating that these genes are within 3.0 centimorgans at the 95% confidence level (43). Similarly, the absence of recombination between *Gdf5* (44) and *Cdmp1* in 125 mice suggests that these genes colocalize within 2.4 centimorgans. This map location suggested close proximity to the brachypodism locus (Fig. 6).

CDMPs Are Predominantly Expressed in Cartilage in Postnatal Life—Northern analyses of a number of postnatal tissues indicated that CDMP-1 could only be detected in newborn articular and cricoid cartilage. In both cases, a single mRNA transcript of approximately 3 kb (Fig. 7) was observed. In contrast, BMP-3 and BMP-7 cDNAs were detected in subsequent hybridizations with the same blots in mRNA of lung, kidney, brain, and small intestine (results not shown) consistent with previous results (15, 17). CDMP-2 mRNA was present in postnatal bovine articular and cricoid cartilage as a 4.6-kb band. After prolonged exposure, some hybridization at 4.6 and 4.0 kb could be detected in mRNA from colon and small intestine (data not shown), skeletal muscle, and placenta (Fig. 7).

CDMPs Are Preferentially Expressed in the Cartilaginous Cores of Long Bones during Human Embryogenesis—The distributions of CDMP-1 and CDMP-2 transcripts in developing limbs were examined in serial sections from fetal tissue representing different stages of early human development. At 6 weeks of gestation, CDMP-1 transcripts were found in precartilage condensations within the developing limbs (Fig. 8, *A-C*). At 7.5–8.5 weeks of gestation, CDMP-1 mRNA expression was found in the cartilaginous cores of long bones, including the articular surfaces (Fig. 8, *D-G*). In areas of active cartilage degradation and bone matrix formation, CDMP-1 expression was also detected in hypertrophic chondrocytes (Fig. 8*H*). Remarkably, no expression was detected in the axial skeleton, and only low mRNA levels were seen in other tissues, such as distal convoluted tubules of the developing kidney, brain, and placenta (data not shown). Immunohistochemical staining indicated that CDMP-1 protein colocalized with the mRNA (Fig. 8, *I* and *J*). However, in addition to the sites of transcription, the protein was also found in the surrounding cartilaginous matrix and in osteoblast-like cells from the primary ossification centers of long bones (Fig. 8*J*).

Between 9 and 10 weeks of gestation, CDMP-2 expression was predominantly localized in the more mature and hypertrophic chondrocytes in regions of invasion by blood vessels through the periosteal bony collar of the developing long bone (Fig. 9). Again, as for CDMP-1, no hybridization was detected in the vertebral bodies in the corresponding sections and stages of human embryonic development (data not shown). Low expression levels were detected in the periosteum (Fig. 9).

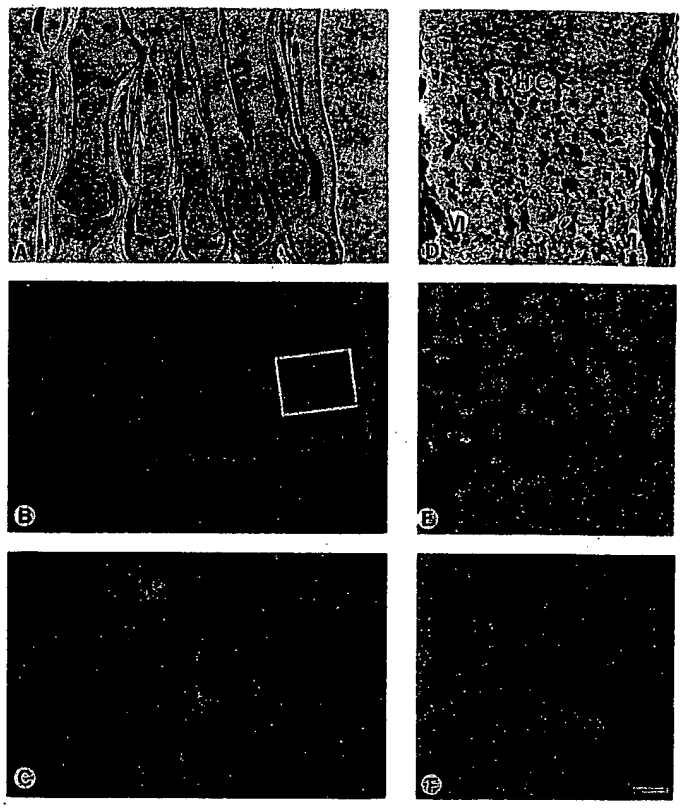

FIG. 9. **CDMP-2 is predominantly localized in hypertrophic chondrocytes of the developing long bones.** Bright field (*A* and *D*) and dark field images of sections through the ossification center of long bones (digits of the foot) in human embryonic development (10 weeks) using an antisense (*B* and *E*) and sense (*C* and *F*) CDMP-2 riboprobe. *E* is the enlarged area of the *boxed zone* in *B*. *D* and *F* are enlarged areas of equivalent zones in *A* and *C*, respectively. *Bar* indicates 500 μm in *A*, 200 μm in *B* and *C*, and 25 μm in *D–F*. *VI*, zone of vascular invasion; *HC*, hypertrophic chondrocytes.

DISCUSSION

In this study, we demonstrate that newborn articular cartilage contains chondrogenic and osteogenic activity when assayed in the *in vivo* subcutaneous implantation model. Using a procedure adapted from that used for the isolation of BMPs from demineralized bone matrix, we partially purified this activity and thereby provided evidence for the presence of BMP-like molecules in cartilage. With degenerate primers designed from conserved regions of known BMPs, we amplified reverse-transcribed mRNA from articular cartilage to identify two novel genes, CDMP-1 and CDMP-2. Both belong to the TGF-β superfamily and are most closely related to BMP-5, BMP-6, and BMP-7 (OP-1). Based on the high percentage identity of their carboxyl-terminal domains, they can be classified as members of a novel subfamily. Although CDMP-1 and CDMP-2 were identified in two different species (human and bovine), they are two distinct genes since the sequences of their pro-regions are significantly divergent (Figs. 2 and 4), as are their expression patterns (Figs. 7 and 8). In contrast to the other members of the BMP family, expression of CDMP-1 and CDMP-2 was predominant in cartilaginous tissues both pre- and postnatally. Such tissue specificity suggests their intimate involvement with skeletal development and growth. If this is correct, mutations of these genes could result in disturbed limb development. Chromosomal mapping of CDMP-1 in mouse indicated a possible link to brachypodism (*bp* mice). This disorder is characterized by a distinct shortening of the limbs without other tissue abnormalities. The defect has previously been attributed to lack of production of a chondrogenic signal by mesenchymal cells at the time of chondrogenesis (45). During the course of our investigation, an independent study (44) described the isolation of the mouse equivalent of CDMP-1, called GDF-5, and established its linkage to the brachypodism mutation. The types of mutations observed in brachypodism mice were found to be effective null mutations for the gene encoding GDF-5/CDMP-1 (44). The pattern of expression of CDMP-1 throughout the cartilaginous core observed during human embryonic long bone development, coupled with the brachypodism mutation in mice (44), indicates that its primary physiological role is most likely at the stage of early chondrogenesis and chondrocyte differentiation in the developing limb.

The expression pattern of CDMP-2, most probably the bovine homologue of the mouse GDF-6 (44), suggests that it is involved in the terminal differentiation of chondrocytes (hypertrophic and mineralizing) and at the earliest stages of endochondral

bone formation, including angiogenesis and osteoblast differentiation. In addition, the relatively high levels of expression (detectable in total RNA blots) in postnatal cartilage suggest possible roles in the maintenance and stabilization of the cartilage phenotype after birth. This is consistent with previous findings, which demonstrated that other family members, BMP-3 (osteogenin) and BMP-4, enhance matrix synthesis *in vitro* in several articular chondrocyte culture systems, thus contributing to the maintenance of the cartilage phenotype (32, 46, 47).

Several BMPs have now been implicated in early skeletal development, including BMP-2, -4, -5, -7, and CDMP-1 (GDF-5). Other members, such as BMP-3, -6, -7, and CDMP-2, may be involved in later stages of skeletal formation (13, 15). The role of the BMPs in early development could be chemotactic, mitogenic, or inductive. Their function in later stages of skeletal development might be promotion of differentiation and maintenance of the established phenotype. The availability of mouse strains with null mutations in specific BMP members, such as the short-ear mice (*Bmp5*) and the brachypodism mice (*Cdmp1/Gdf5*), allows analysis of the specific contributions of the respective members in each of the stages of skeletal development. Similarly, transgenic approaches may well provide more insight with regard to the role of other BMPs in development.

The absence of expression of both CDMP-1 and CDMP-2 in the axial skeleton has implications for models of skeletal development; the brachypodism mice, for example, have disturbed limb development but a normal axial skeleton. This is the first evidence that the developmental mechanisms and differentiation pathways of the vertebral bodies are distinct from those of the peripheral skeletal elements and indicates that the basic form and pattern of the skeleton are likely to be determined by a number of BMP-like signaling molecules. The discovery of this novel subgroup of BMP-like cartilage-specific molecules provides additional tools to study the regulation of the differentiation of the chondrocytic lineage and provides new opportunities for regeneration of cartilaginous tissues in clinical contexts.

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